Isolation, Identification and biochemical analysis of Mercury Resistant Bacteria (MRB) from the effluent water of Rourkela Steel Plant, Orissa

DISSERTATION PROJECT SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTERS OF SCIENCE IN LIFE SCIENCE

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CERTIFICATE

This is to certify that the project report titled **"Isolation, Identification and Biochemical analysis of Mercury resistant bacteria (MRB) from the effluent water of Rourkela Steel Plant, Orissa"** submitted by Ms Sony Priyadarshini to the department of Life Sciences, National Institute of Technology, Rourkela in partial fulfillment of the requirements for the degree of Masters of Science in LIFE SCIENCES is a bonafide record of work carried out by her under my supervision. The contents of this report in full or parts have not been submitted to any other Institute or University for the award of any degree or diploma.

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1. INTRODUCTION

Microbial world is invisible to unaided human eyes. Thus what goes on in their domain is not easily perceived, unless some direct measurements and analyses are carried out. Comprising of bacteria, fungi, yeasts, protozoans, phytoplankton and other micro fauna within 500 μm size, microbial communities perform immense tasks mainly to keep themselves perpetuating in their ecosystems. Though it is for their survival, growth and reproduction, the microbial activities of photosynthesis (by microscopic phytoplankton), (respiration by all living beings) and breaking down of organic matters into simpler moieties and finally to inorganic molecules by all heterotrophic beings are the pivotal roles that help the earth's ecosystems function and attain, as far as possible a dynamic equilibrium (Barkay et al., 2003). On the whole, sunlight is the only external input the life on earth needs. All other matter is produced, consumed and recycled by an array of organisms inhabiting the earth in her varied and often extreme habitats. In essence, the environmental functioning and stability are continuously aided and maintained by microorganism's activities.

Any imbalance largely due to their activities through human natural effects adversely affects the ecosystems. Pollution due to anthropogenic activities is the greatest problem all the ecosystems were subjected to right from the beginning of human dominance through the hunting-gathering, settled agricultural to modern industrialized civilizations. In the last two millennia or so, the human population growth and indiscrete uses of earth's non-renewable resources have brought about rapid changes to the extent that there are already innumerable degraded/retarded habitats spoiling the Mother Earth rather undesirably. The long term consequences of retarded habitats are too numerous including shifts in human settlements, societal conflicts, diseases, economic losses, community and species diversity shifts, global changes and health effects. The one "adjustment" natural organismic community makes in the face of all and life-threatening ill effects of pollution in their metabolic potential are modification in its life-style so much so that some of its representatives "go-on". This adjustment can be termed variously as tolerance or resistance (Osborn et al., 1997). Many components of organismic communities have the potential for such adjustment. In industrial area as both

metal and organic pollution is of vital concern, interest in bacterial resistance to metal salts especially when associated with degradative activities is of practical significance. Though studies on tolerance to toxic metals and degradative capacities have been extensive, these two aspects are often addressed separately. Often they have not taken into account that in industrial areas organic pollution (fossil fuels or their derivatives, pesticides, PCBs and TBT among others) is often accompanied by inorganic ones mainly of heavy metals (mercury, cadmium, lead to name a few). Without efficient retention technologies, toxic chemicals including Hg are let into the environment, endangering ecosystems and public health.

1.1. Mercury in the environment:-

Mercury, the only metal in liquid form at room temperature is the most toxic of the heavy metals (Gerlach, 1981) and the sixth most toxic chemical in the list of hazardous compounds (Nascimento and Chartone-Souza, 2003) has been present in the environment for years. Erupted from the core of earth by volcanic activity it exists as mineral (mostly as cinnabar-HgS), as mercuric oxide, oxychloride, sulfate mineral (Kim et al., 2001) or also as elemental mercury. It also exists as gas due to its high vapor pressure (Fig. 1).

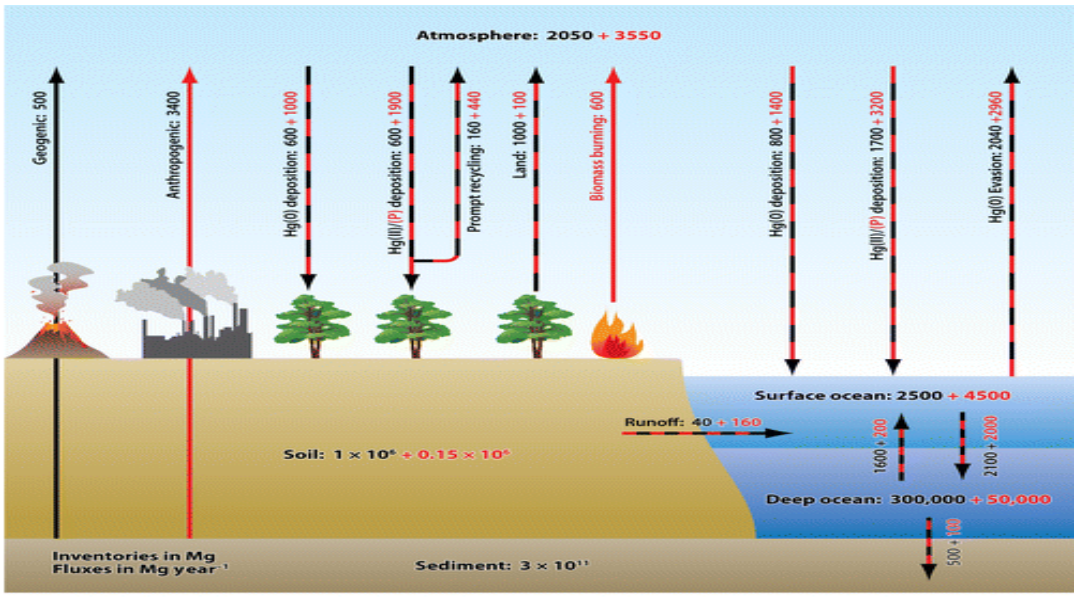

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Figure 1. Different forms of mercury in the environment augmented by anthropogenic activities.

In a biogeochemical cycle (Fig. 2) mercury is globally dispersed undergoing many physical and Chemical transformations (Barkay et al., 2003):

i) In the atmosphere elemental mercury is photo-oxidized to ionic mercury (Hg^{2+}).

ii) Rain precipitates the inorganic mercury on the surface of the earth, where carried out mainly by microorganisms in aquatic systems,

iii) it is reduced back to its elemental form or

iv) methylated.

v) elemental mercury evaporates into air where the cycle begins a new.

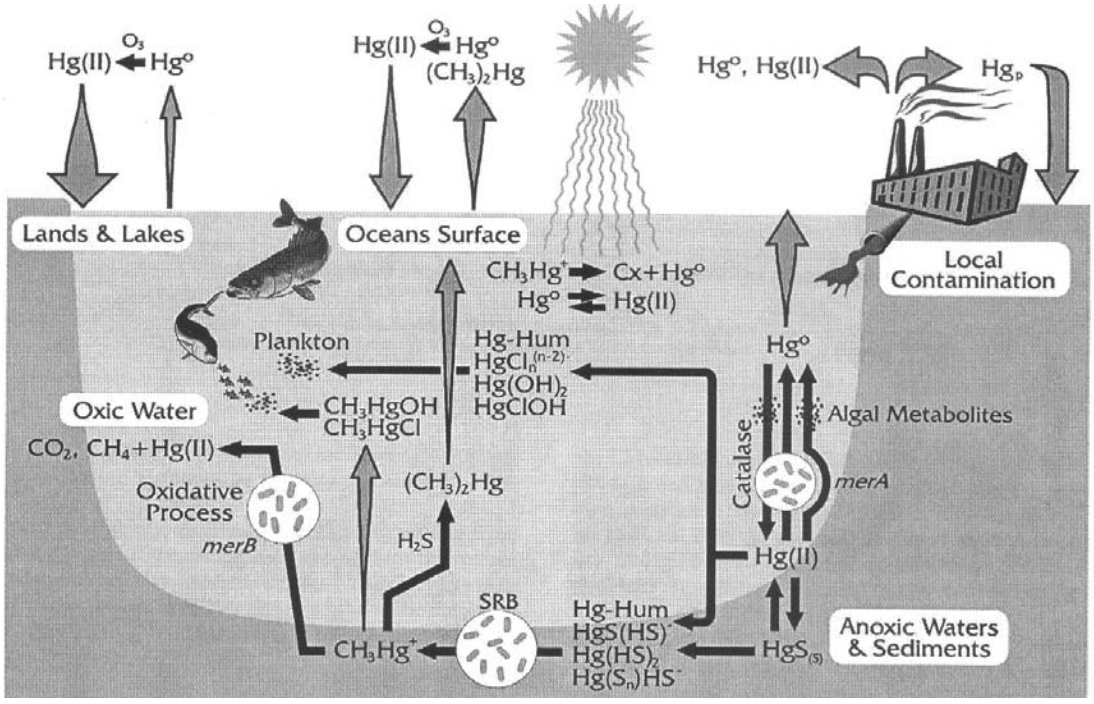


Figure 2. The biogeochemical cycle of mercury in the environment (Barkay et al., 2003). Solid arrows represent transformation or uptake of mercury. Hollow arrows indicate flux of mercury between different compartments in the environments. The width of the arrows is approximately proportional to the relative importance of the flux in nature. Speciation of Hg(II) in oxic and anoxic waters is controlled by chloride and hydroxide, and by sulfide respectively. Circles depicting bacterial cells represent transformations known to be mediated by microorganisms. A group of dots indicate the involvement of unicellular algae. Light-mediated water column transformations positioned below the sun. Photodegradation of CH3Hg+ results in mostly Hg0 and an unknown C1 species depicted as Cx.

The four main natural processes (Fig .3)that aid Hg emission are:

i) degassing from geological mineral deposits,

ii) emissions from volcanic activities,

iii) photoreduction of divalent mercury in aquatic systems and

iv) biological formation of elemental and methyl mercury.

Mercury has been recognized as one of the most toxic heavy metals in the environment and has been released into the environment in substantial quantities through natural events and anthropogenic activities .Industrial dumping of mercury into rivers , the consumption of coal and solid waste incineration has led to significant pollution of the environment (Von Canstein et al., 2001).Mercury binds to the sulfahydryl groups of enzymes and proteins , thereby inactivating vital cell functions (Wagner Dobler et al.,2000a). Entrance of the most toxic species of mercury i.e. methyl mercury into the human body results in Minamata disease. Different neurological effects such as paresthensia and numbness in the fingers are common symptoms of Minamata disease (UNEP, 2003).

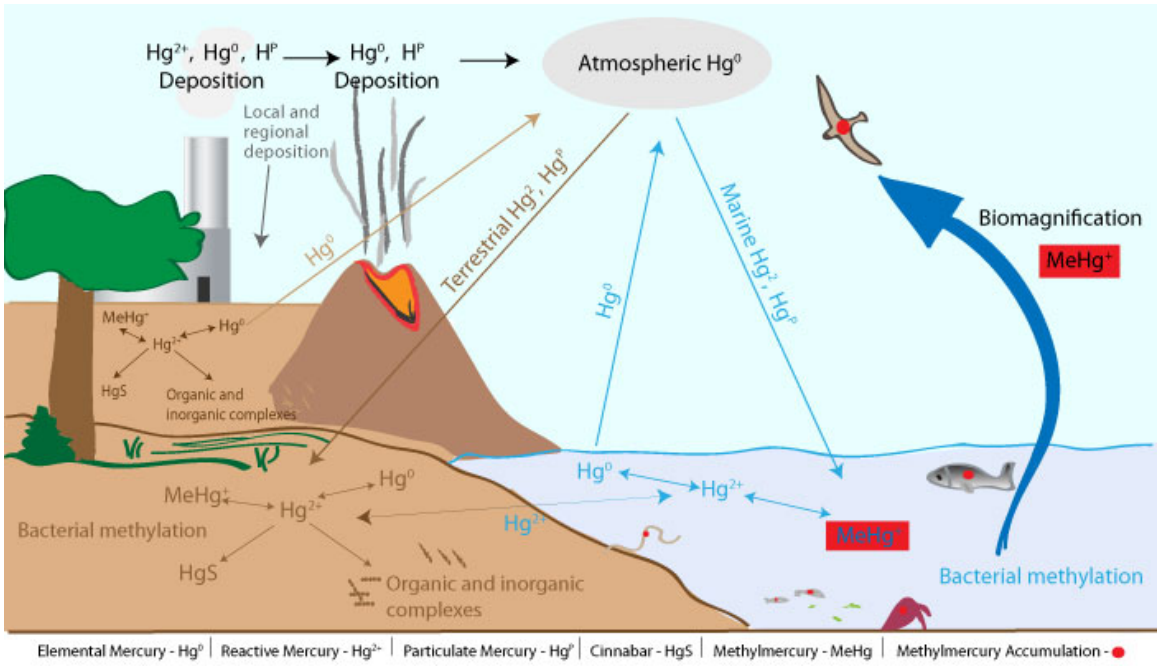


Figure 3. Processes leading to mercury emission

Although it is undisputed that mercury occurs naturally and toxic concentrations in some locations, mercury emissions owing to anthropogenic activities (mainly through chloralkalielectrolysis and chlorine production), mining and fossil fuel combustion or waste incineration are immense, contributing substantially to the mercury pool participating in the biogeochemical cycle. However, the concentrations of mercury in various compartments from natural and anthropogenic sources are highly variable. Recent measurements of mercury in aquatic systems have given the following concentration ranges: open ocean (0.5-3 ng l^{-1}), coastal seawater (2-15 ng l^{-1}), rivers and lakes (1-3 ng l^{-1}). These may be considered representative for dissolved mercury (http://www.inchem.org/documents/ehc/ehc/ehc086.htm#SectionNumber:3.3). Recently, the stringent legislation in the US or European countries have brought down the anthropogenic input and the global mercury estimate is on the decrease (Slemr, 2003). Mercury is widely used in industry because of its diverse properties like it is odorless, liquid at room temperature, tolerates high temperature and is highly volatile in nature.

Worldwide many areas contaminated with mercury pose threat to people and environment (Xiao et al., 1998; Cleckner et al., 1999; Fukuda et al., 1999; Horvat et al., 1999). Owing to the mercury cycle described earlier, regional emissions may be deposited elsewhere, in far off uninhabited regimes, for e.g., in the Arctic (Macdonald et al., 2000). Hence local, regional and small-scale contaminations do not remain confined to a particular area but affect ecosystems globally.

Mercury (mostly as methylmercury) accumulates in carnivorous fish via the food chain by a process called biomagnification and poison people consuming fish. The syndromes such as neurological disorders, resulting from the poisoning have been named Minamata disease in the late 1950s when over 3000 people around this Minamata Bay in Japan were severely poisoned by methylmercury pollution caused by a chemical manufacturing plant releasing its effluents containing mercury (Langford and Ferner, 1999).

1.2. Toxicity of mercury:-

Mercury was widely used in clinical thermometers before the prohibition by the European Union Directive (European Council Directive 93/42/EEC). Moreover, it can be found in several medications and is still used in dental fillings. However, its toxicity has also been known and recorded for two millennia (Langford and Ferner 1999). Different

forms of mercury possess different characteristics rendering it a hazard to living organisms. The toxicity of highly reactive mercuric mercury (Hg^{2+}) is attributed to its binding to the sulphydryl groups, of the cysteines of essential enzymes and proteins, thus disturbing vital cell functions (Fig. 4). In the body water soluble ionic mercury salts are not efficiently absorbed. Rather, they are eliminated from the body via kidneys acutely causing damage to the gut and the renal systems. The hazard arising from elemental mercury (Hg^0) is owed to its high vapour pressure allowing it to be easily inhaled. Absorbed by the lungs it enters the blood and circulated throughout the body including the brain. Elemental mercury is transformed in the red blood cells (RBC), the liver and central nervous system (CNS) to Hg^{2+} and methyl mercury. Repeated or prolonged exposure mainly results in vasomotor disturbances, tremor and behavioural disturbances. Organic mercury compounds like mono or di methylmercury or phenylmercuric acetate (PMA) are lipid soluble and thus readily absorbed into the body. They penetrate membranes and cross the blood-brain barrier. A large proportion of organic mercury is transformed into reactive Hg^{2+} (Strasdeit, 1998) and can severely damage the CNS causing neuromuscular malfunctions, ranging from numb limbs, visual disorders to paralysis and even death (Barkay 2000). The clinical and epidemiological evidences indicate that the prenatal life is more sensitive to toxic effects of methylmercury than adults which results into abnormal neuronal development, leading to altered brain architecture and brain size. Because transformation to Hg^{2+} occurs slowly, symptoms of poisoning with organic mercury may appear weeks or months later to the initial poisoning as was detected in the case of Karen Wetterhahn, a chemistry professor (Nierenberg *et al.,* 1998).

Today, the major source of human Exposure to mercury is the consumption of seafood (Boudou and Ribeyre, 1997). Mercury compounds are acutely toxic to freshwater microorganisms. The no observed- toxic-effect-level (NOTEL) for inorganic mercury lies between 1-50 ppb (µg/l) depending on the organism, density of the cell in culture and experimental conditions. Diversity of species in a mixed culture may be affected by 40 ppb $HgCl_2$ (Canstein, 2000). For organic mercury, the NOTEL is 10-100 times lower. Aquatic plants sustain damage after exposure to inorganic mercury at concentrations of

800-1200 ppb. Organomercury exerts toxicity at concentrations 10-100 times lower. Many aquatic invertebrates, particularly larvae are sensitive to mercury toxicity. For the most sensitive species, *Daphnia magna*, the NOTEL for reproductive impairment is 3 ppb for inorganic mercury and lesser than 0.04 ppb for methylmercury (Canstein, 2000). Hence it is of great importance for both environment and public health to avoid mercury contamination/discharges into freshwater and marine ecosystem.

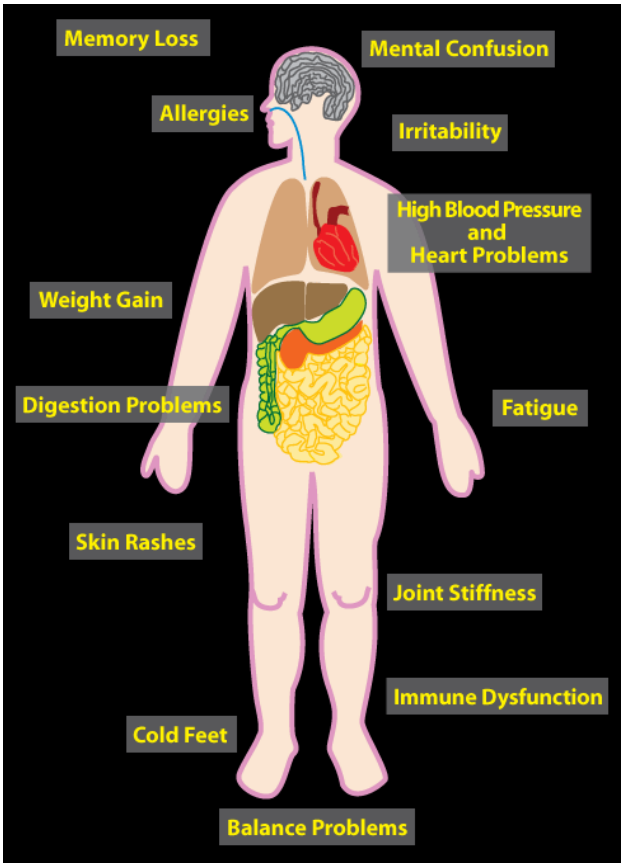


Figure 4. Toxicity of mercury towards Human body

1.3. Bacterial resistance to mercury:

As a response to toxic mercury compounds globally distributed by geological and anthropogenic activities, microbes have developed a surprising array of resistance mechanisms to overcome Hg toxicity. An extensively studied resistance system based on clustered genes in an operon (i.e.*Mer*) (Fig. 5), allows bacteria to detoxify Hg^{2+} into volatile mercury by enzymatic reduction (Komura and Izaki, 1971; Summers, 1986; Misra, 1992; Silver, 1996; Osborn et al., 1997; Barkay et al., 2003).

It appears that bacterial resistance to mercury is an ancient mechanism, probably acquired even before anthropogenic usage of mercury. Since the same bio transformations that constitute the Hg biogeochemical cycle can take place inside the human body, understanding its external transformations and transport processes will help in figuring out which of these processes can exacerbate or ameliorate Hg toxicity in humans (Barkay et al., 2003).

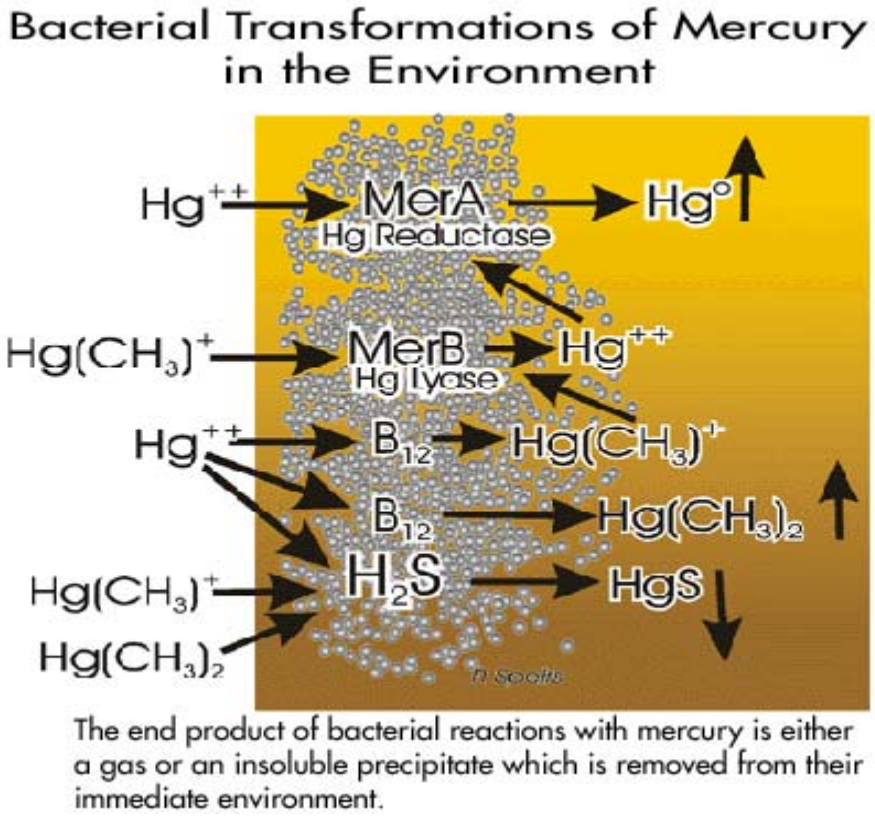


Figure 5. Bacterial transformations of mercury in the environment

Mercury-resistance determinants have been found in a wide range of Gram-negative and Gram positive bacteria isolated from different environments. They vary in number and identity of genes involved and are encoded by the *mer* operon located on plasmid (Summers and Silver, 1978; Brown et al., 1986; Griffin et al., 1987; Radstrom et al., 1994; Osborn et al., 1997), chromosomes(Wang et al., 1987, 1989; Inoue et al., 1989, 1999; Iohara et al., 2001), transposons (Misra et al.,1984; Kholodi et al., 1993; Liebert et al., 1997, 1999; Hobman et al., 1994) and integrons (Liebert et al., 1999).

Mercuric mercury complexes with organic and inorganic ligands and, is easily adsorbed to surfaces of particulates owing to its high reactivity and affinity to thiol groups (Barkay, 2000). Mercury bioavailability therefore plays a crucial role in the evaluation of microbial resistancelevels.

There is some tolerance towards mercury owing solely to unspecific sequestration by cell walls and lipopolysaccharide (LPS) layers (Langley and Beverudge, 1999).
The narrow mercury resistance operon (*mer*) comprises three major functions:-

a) transport of Hg^{2+} into the cell,

b) enzymatic NADPH-dependent conversion of the ionic mercury into relatively less toxic elemental mercury (Hg^0) and

c) regulation of the functional genes (Misra, 1992; Silver and Phung, 1996).

Detoxification of organic mercury requires a fourth function, namely the cleavage of mercury from the organic residue and the resistance is termed as "broad spectrum". Genes conferring these functions are designated as *mer*T, *mer*P (transport), *mer*A (mercury reduction), *mer*B (cleavage of Hg from organic residue), *mer*R and *mer*D (regulation). Further, more of *mer* genes have been identified recently; *mer*C and *mer*F, both membrane proteins, conferring transport functions (Wilson et al., 2000). Also *mer*G is known to confer resistance to phenylmercury (Kiyono and Pan Hou, 1999). Most mercury resistance operons are inducible, i.e. Hg^{2+} has to be present in order to activate expression of resistance whereas transcription is suppressed in the absence of mercury (Misra, 1992).

If, however, switched on by mercury at a contaminated site, the bacterial mercury resistance forms the basis of natural on-site detoxification (Silver et al., 1994) under aerobic conditions. Mercury pollution can contribute to increased antibiotic resistance (McArthur and Tuckfield, 2000). The combined expression of resistance to antibiotic and mercury may be caused by selection, as a consequence of the mercury present in an environment (Sant'ana et al., 1989). Many researchers have claimed that Hg-reductase activity in Hg-resistant bacteria is always inducible and never constitutive (Summers, 1986). However, Nakamura et al. (1986) showed the presence of a constitutive Hg-reductase in *Streptomyces* sp. Induction of the *mer* operon has been proposed to take place when the concentration of mercury exceeds50 ppM, microbial reduction via *mer*A

reductase likely becomes the predominant mechanism of Hg (II) detoxification (Morel et al., 1998).

1.4. Mercury-resistant bacteria in bioremediation:

The ability of bacteria to detoxify mercury can be utilized to bioremediate mercury contaminated wastewater. In fact, it seems as if this potential was solely confined to microbial species as non naturally occurring plant or animal species have been reported to detoxify mercury. Nevertheless,plants have been engineered to overexpress the bacterial mercury resistance and transform organic and inorganic mercury to elemental mercury with promising result for phytoremediation of mercury-contaminated sites (Rugh et al., 1998; Bizily, 1999). Plants combine many practical benefits for the bioremediation of contaminated sites. With the mercury transforming transgenic yellow poplar (Rugh et al., 1998), large areas of contamination could be dealt with. In addition to

decontamination, these with stout-root trees would stabilize the soil surface and provide niches for the inhabitation of mercury- reducing microbes.

However, the mercury reducing plants emit volatile Hg^0 in to the air, which although non-significant, on a global scale, may contribute to increased mercury concentrations locally. One of the initial efforts to retain mercury in bacterial bioreactors was made by (Brunke et al.1993). They managed to capture elemental mercury in globules upto 5 μm in diameter in fixed bedcolumns using genetically engineered mercury-reducing bacteria that were immobilized on ceramic carriers, glass or in alginate beads. Some years later, (Canstein et al. 1999) demonstrated the removal of mercury from chloralkali electrolysis wasterwater by a mercury resistant *Pseudomonas putida* strain. This natural isolate was capable of coping with upto 8 ppm of mercury in the wastewater, transforming >97% (when cellulose fibers were the carriers) or 98.5% (with siran as the carrier). These laboratory-scale reactor results formed the basis for the development of a technical scale bioreactor that decontaminated mercury-polluted chloralkali wastewater *in situ* (Wagner-Döbler et al., 2000, 2003).

Mercury retention could be achieved upto 95% efficiency, and the discharge limit for mercury in industrial wastewater (50 ppb) could be met reliably with the help of an activated carbon filter at the end of the line, capturing residual traces of mercury. The

elemental mercury accumulated in the bioreactor, this however, did not affect the efficacy of the bioreactor.It was found that although inoculated with several mercury resistant isolates, foreign mercuryresistant bacteria invaded the reactor and a new consortium of mercury-transforming bacteria evolved that dynamically changed over time (Canstein et al., 2001). In experiments in the laboratory-scale bioreactors, the presence of a consortium could be shown to be of benefit for a reliable, disturbance-independent mercury-removal (Canstein et al., 2002). Due to the continuous selective pressure in the bioreactor, MRB that are most properly adapted to onsite conditions are enriched and this is best suited to improve bioreactor stability.

2. AIMS & OBJECTIVES

- To characterize mercury resistant bacteria populations & THB populations.
- To understand the mechanism of resistance to mercury.
- To find out the Minimum Inhibitory Concentrations.
- To carry out biochemical characterization & antibiotic sensitivity.
- To study the level of gene expression in the resistant strains .

3. PLAN OF WORK

Collection of water sample from four different sites (Lagoon Inlet) of RSP, Rourkela.

↓

Enumeration of THB (Total heterotrophic bacterial count) and MRB (Mercury Resisitant bacterial count).

↓

Minimum Inhibitory Concentration (MIC Test)

↓

Selection of MRB showing highest MIC

↓

Antibiotic Susceptibility Test

↓

Biochemical Tests for characterization

4. REVIEW OF LITERATURE

Comprising <0.5 to 4 μm sized single cells, bacterial communities are of immense importance both for ecosystem function and stability. Most uniquely this prokaryotic life can develop, adapt and survive in the most inhospitable surroundings. Their physiological responses such as viability (Colwell et al., 1985; Oliver et al., 1995; Naganuma, 1996), metabolism, resting stages and death (Amy and Morita, 1983) are caused by certain adverse anthropogenic and some natural processes. As natural assemblages experiencing the 'wilderness' imposed through pollution by anthropogenic expansionism, bacteria adapt quite rapidly and gear up themselves to cope up with adversities, rather successfully, to a very great extent. Monitoring bacterial responses of any type is useful for assessing marine microbial heterotrophy (Kogure et al., 1987) and environmental quality (Liebert and Barkay, 1988; Ramaiah & Chandramohan, 1993; Ramaiah et al., 2002a, 2002b). Thus, for a long time now, many investigations have been using bacterial indicators (e.g., coliform groups) to assess effluent discharges into the coastal/ marine environment. Long-term exposure to heavy metals viz. Zn, Cu and Ni (Bååth et al., 1998) and Hg (Rasmussen and Sørensen, 1998, 2001; Müller et al., 2001a) has been found to alter the microbial community.

Observations on occurrence and distribution of native bacteria capable of metal tolerance are of relevance in microbial ecology to understand the extent of metal pollution (Rasmussen and Sørensen, 2001; Müller et al., 2001b) and to realize the potential of such flora in detoxifying toxic substances (Barbieri et al., 1996; Ka et al., 1994; De et al., 2003). Many human activities have negative impact on several biological processes and there is no doubt that these will continue to affect the functioning of highly productive coastal ecosystems. Contamination caused by heavy metals like mercury affects both oceanic and those of continental shelf and coastal regimes, along with waste water effluents from the industries where besides longer residence times, metal concentrations are higher due to the anthropogenic input, transport by river runoff and the proximity to industrial and urban zones (Nriagu, 1990; Rainbow and Furness, 1990).Many studies on the biota, sediments and water have reported concentrations far above the levels tolerable by humans (Knight et al., 1997; Moreira and Piveta, 1997; Olivero and Solano, 1998; Bastidas et al., 1999a, 1999b). Leaching naturally (Gerlach,

1981; Anon, 1998) and added anthropogenically, mercury (Hg) keeps accruing in seawater and marine sediments. Passive uptake of Hg and its compounds by many osmotrophs including bacteria leads to their modification and in changed forms, they move up the food chain, get bio-magnified and reach human beings where they produce chronic and/or acute ailments (Förstner and Wittmann 1979). In addition to natural processes of bedrock leaching, Hg and its compounds enter aquatic bodies by washings from soils and atmosphere (Mason et al., 1994), effluents from chloro-alkali production units (Colwell et al., 1985; Anon, 1999; Nahida et al., 2000) chemical laboratories, instrument manufacturing, dentistry and fluorescent light bulb breakage (Hütter (1978) and from sewage treatment facilities (Soldano et al., 1975). Our current understanding is that due to very slow biogeochemical remobilization, toxic heavy metals (e.g., Hg, Cd, Cu, Pb) and metalloids (e.g., As) forming complexes with organic components of marine sediments (Förstner and Wittmann, 1979; Gerlach, 1981; Barkay,1987; Gilmour et al., 1992) tend to accumulate leading ultimately to deleterious situations to marine life and in turn, human beings. Thus, environmental effects of metal pollution are all encompassing (Baldi et al., 1989).Worldwide many areas are mercury polluted and present a threat to people and environment (Fukuda et al., 1999; Horvat et al., 1999). As a consequence, mercury-resistance is often seen to be associated with the natural flora (Pahan et al., 1990; Canstein, 1999; Macalady, 2000; Müller et al., 2001b; Ramaiah & De, 2003). Recently, stringent legislation in the US and European countries have brought down the anthropogenic input and, consequently, the global mercury estimate is on the decrease (Slemr, 2003).

The toxicology and environmental behaviour of mercury is quite complex, since the toxicity, mobility and bioaccumulation of mercury depend on its chemical form (D'Itri, 1990). Mercury ore, cinnabar (HgS), poses a limited direct threat because of its low solubility (Morel and Hering, 1993). However, under toxic conditions –as existing in surficial sediments, soils and in most surface waters – HgS can be converted to dissolved divalent mercury (Hg^{2+}),elemental mercury (Hg^{0}) and methyl mercury (CH_3Hg^{+}; Klaassen et al., 1986) latter being the most toxic, which created havoc in the early 1960s in Japan resulting in the Minamata disease. Humans are exposed to methyl mercury principally through the consumption of marine fish and fish products, yet research on Hg

transformations and mobility in the marine environment is sparse. Environmental studies have focused on terrestrial regions, especially freshwater ecosystems. In contrast, attention to estuaries and adjacent coastalwaters that are major repositories for natural and river biorne/watershed derived Hg species is scanty. There is a vital need to increase our knowledge and understanding concerning the biogeochemical cycling of Hg and the impact of anthropogenically related inputs in biologically productive near shore regions (Fitzgerald et al., 2000).

Mercury-resistant bacteria (MRB) are widely distributed and quite ubiquitous in nature accounting ca. 1-10% of aerobic heterotrophic bacteria (Müller, 2001a). They can be isolated without prior enrichment. However, resistant strains are more abundant in mercury-polluted environments, where up to 50% may grow on nutrient agar media amended with as high as 50 μM (10 ppm) Hg^{2+}. Whereas, sensitive strains can at best tolerate 1 μM in the growth media (Barkay, 2000). The presence of MRB is often correlated with the level of mercury contamination in an environment, although they have been isolated from uncontaminated environments (Osborn et al., 1993).

Although research on mercury resistance and bacteria is into the fifth decade, there have been very few reports on MRB from the freshwater environments (Garcia et al., 1999). Further, in an open system like oceans, mercury vapour released by resistant bacteria will become part of the local mercury cycle and repollute the environment as has been reported in case of the Amazon river basin (Lacerda Pfeiffer, 1992).

Mercury (Hg) pollution of soil and water is a world-wide problem (Dean et al., 1972; Kramer and Chardonnens, 2001). The extent to which Hg is harmful depends on the form of mercury present in the ecosystem. Mercury has been released into environment in substantial quantities through natural events and anthropogenic activities (Kiyono and Pan-Hou, 2006). Mercury and its compounds when released into the environment are highly toxic to living cells because of their strong affinity for the thiol groups of proteins (Hajela et al., 2002). Industrial use of mercury led to the pollution of environment. Even small amounts of mercury are considered to be toxic for most of the organisms. Consequently, mercury removal is a challenge for environmental management. Microorganisms in contaminated environments have developed resistance to mercury and are playing a major role in natural decontamination. Some bacterial

communities residing in the mercury contaminated areas can exchange mercury resistance genes with each other because of continual exposure to toxic levels of mercury. After the acquisition of these resistance genes these bacteria develop resistance to mercury (Nascimento and Souza,2003). An extensively studied resistance system, based on clustered genes in an operon (mer operon), allows bacteria to detoxify Hg2+ into volatile metallic mercury by enzymatic reduction (Komura et al., 1971; Summers, 1986). Mercury-resistance determinants have been found in a wide range of gram-negative and gram-positive bacteria isolated from different environments.

They vary in the number and identity of genes involved and is encoded by meroperons, usually located on plasmids (Summers and Silver, 1972; Misra, 1992; Brown et al., 1986; Griffin et al., 1987) and chromosomes (Wang et al., 1987; Inoue et al., 1991); they are often components of transposons (Misra et al., 1984; Kholodii et al., 1993) and integrons (Liebert et al., 1999). A widely employed mechanism of bacterial resistance to mercurial compounds is the reduction of Hg^{2+} to its volatile metallic form Hg^0 (Libert et al., 1997). The biotransformation is mediated by mercury reductase, an inducible NADPH-dependent, flavin containing disulfide oxidoreductase enzyme. The gene coding for mercury reductase is merA (Scott et al., 1999). The bacterial mer operon encodes a cluster of genes involved in the detection, mobilization and enzymatic detoxification of mercury. Ionic mercury (Hg^{2+}) is transported into the cytoplasm by a set of transport genes, where the merA gene, which encodes mercuric ion reductase, reduces this highly toxic ionic mercury (Hg^{2+}) to the much less toxic volatile Hg^0. Researchers developed bioremediation as one feasible way to accelerate or encourage the degradation of pollutants.Mercury resistant bacteria were first isolated from mercury contaminated soil in Japan (Robinson and Tuovinen, 1984).After this finding there were several reports of environmental bacteria which were resistant to mercury compounds (Jaysankar *et al.*,2006; Chiu et al*.,* 2007).

Bioremediation can be used to clean unwanted substances from air, soil, water and raw materials from industrial processing. Hyper-accumulation and hyper tolerance of Hg is the characteristic of few plants but they have not shown the ability to detoxify the toxic form of Hg to non-toxic form (Lenka et al*.,* 1990. These compounds can pass through biological membranes (Gutknecht 1981) and bind with high affinity to thiol (SH)

groups in proteins, thus causing damage to membranes and inactivating enzymes. Mercury is also genotoxic; inorganic Hg(II) is capable of strong reversible interactions with the nitrogens in purines and pyrimidines, and organic mercury compounds, e.g. methylmercury, also produce irreversible damage to nucleic acids (Sletten and Nerdal, 1997).

Environmental contamination with mercury compounds can have devastating effects as mercury toxicity is cumulative, with the highest levels of mercury compounds being found in consumers at the top of the food chain.A number of microorganisms have evolved resistance mechanisms to deal with mercury compounds. Mercury resistance was first reported in Staphylococcus aureus (Moore, 1960) and since then has been described in a number of bacterial species. One of the best defined mercury resistance determinants is the mer operon encoded by transposon Tn501, found in Gram-negative bacteria. The functions of the minimal number of proteins required to confer full resistance are as follows (Hobman and Brown 1997): MerR is the mer regulatory protein which controls the expression of all the other proteins in the operon in response to the presence of Hg(II) salts. MerP, the periplasmic Hg binding protein, transfers Hg(II) to the MerT transport protein located in the cytoplasmic membrane. This passes mercuric ions to the cytoplasmic mercuric reductase (merA gene product), which reduces Hg(II) to Hg(0) using NADPH as the reductant. Hg(0) is then lost from the cell in the gas phase.Mercuric ion resistance has also been characterized in Gram-positive genera. The resistance determinants from Bacillus sp. RC607 (Wang et al. 1989), Streptomyces lividans (Sedlmeier and Altenbuchner, 1992) and S. aureus (Laddaga et al., 1987) have been characterised.

The mechanisms of induction, mercury transport and mercury reduction in Gram-positive genera are similar to those of the Gram-negative systems, but the transport functions differ, as might be expected from the differences in the surfaces of Gram-positive and Gram-negative cells (Hobman and Brown 1997). Although resistance to Hg(II) has been widely investigated, there are few reports of mercuric ion resistance in extremophilic microorganisms (Olson et al., 1982, Bogdanova et al., 1988). Given the widespread occurrence of mercuric ion resistance/ tolerance throughout the bacterial kingdom, it seems likely that many extremophilic microorganisms will also encode

mercury resistance. This communication describes the investigation of such resistance with the aim to develop methods for mercury bioremediation which could be accelerated by using higher temperatures than, for example, well published examples such as the Hg(II) reduction system based on the reduction of Hg(II) to Hg(0) (Brunke et al., 1993). As a first step we report the isolation of a thermophilic *Bacillus* sp. (Scholz et al., 1987) and *Ureibacillus* sp. (Fortina et al., 2001) and investigate their observed mercuric ion resistance. Mercury resistance ability has not only been reported in bacteria but in different archeal species too (Schelert et al., 2004 and Vetriani et al., 2004).

It is now quite an established fact that elevated levels of mercury, whether by natural processes or by anthropogenic input, exerts selection pressure on microbial communities. Those communities that can be adapted are going to be the ones that will live through in environments contaminated by this toxic heavy metal. Many species of bacteria have developed several ways to cope up with mercury toxicity. A detailed and all compassing review by Osborn et al. (1997) lists uptake of mercuric ions (Hg^{2+}) despite low cellular permeability, demethylation of methyl mercury followed by conversion to mercuric sulfide compounds, sequestration of methyl mercury by continuous production of hydrogen sulfide, methylation of mercuric mercury to the volatile methylmercury, and the enzymatic reduction of Hg^{2+} to Hg^{0} as the major bacterial mechanisms to deal with Hg toxicity.Resistance against mercury compounds, mediated by the microbial *mer* operon was discovered in the early 1970's (Summers and Lewis, 1973). Since then, several "archetypical" *mer* operons(Brown et al., 1991; Liebert et al., 1999) have been studied in depth with respect to structure, function and regulation of the individual gene products (Ji & Silver, 1995; Nies, 1999; Barkay et al., 2003).

Genes conferring resistances to mercury compounds are clustered in an operon in most known naturally occurring systems (Silver and Phung, 1996; Barkay et al., 2003). The *mer* resistance components can be subgrouped into three categories based on their functional roles:

a) transporters of Hg(II) into the cells,

b) converters (enzymatically) of toxic mercury compounds into a relatively nontoxic form [Hg(0)] and

c) regulators of operon expression.

The *mer* operons from both gram-positive and gram-negative bacteria have been cloned and sequenced. Genetic and biochemical studies have advanced our knowledge, leading to in- depth understanding of resistance mechanism, gene evolution, and regulation of expression of the *mer* genes (Misra,1992). This energy consuming reaction is based on the ubiquitous *mer* operon. Most *mer* operons contain atleast the mercury-resistance genes *mer*R, *mer*D, *mer*T, *mer*P and *mer*A (Silver and Phung, 1996; Osborn et al., 1997). Expression of *mer* operon is regulated by the products of *mer*R and *mer*D and is inducible by Hg (II). The product of *mer*R represses operon expression in the absence of inducer and activates transcription in the presence of inducer. The product of *mer*D coregulates expression of operon (Misra, 1992; Silver and Phung, 1996). Products of periplasmic *mer*T and inner membrane-*mer*P take part in the transport of metal across the cell membrane. Products of *mer*C and *mer*F, both membrane proteins were found to act as mercury transport system (Kusano et al., 1990; Wilson et al., 2000). Bacteria that are resistant only to Hg(II) have a so-called "narrow spectrum resistance", whereas others that are resistant to both Hg (II) and certain organomercurials are with broad-spectrum resistance. Resistance against organomercurials depends on the organomercurial lyase MerB that cleaves the carbon-mercury bond of the organomercurials, and the resultant product Hg (II) can be subsequently reduced by the mercuric reductase (Silver and Misra, 1988). In some cases, the *mer* operon contains other functional genes. The *mer*G product provides phenylmercury resistance, presumably by reducing the in-cell permeability to phenylmercury (Kiyono and Pan-Hou, 1999).

As expression of these genes is induced with low levels of mercury salts, Hg(II) functions as an environmental sensor, switching on the synthesis of mercury resistance protein components. Both merC and merT contain two pairs of cysteine and periplasmic merP contains one pair of cysteine. It is hypothesized (Misra, 1992) that these cysteine pairs (thiol groups) sequester Hg(II) and transfer Hg (II) to the thiols of cytoplasmic mercuric reductase via the transmembrane protein MerT. Thus Hg(II) is never free to interact with cellular constituents, and only the elemental form of mercury [Hg(0)] is released in the cytoplasm. Mercuric reductase (product of *mer*A) is the key enzyme in reduction and detoxification of Hg (II). It is a flavin adenine dinucleotide (FAD) - containing disulfide oxidoreductase that transfers electrons of NADPH or NADH to Hg

(II). The resulting metallic mercury Hg (0) is harmless to bacteria and diffuses out of the cell (Miller et al., 1986; Misra, 1992; Silver and Phung, 1996; Hobman and Brown, 1997; Osborn et al., 1997). In aerobic environs, metallic mercury volatilizes and thus the cell detoxifies its surrounding microenvironment.. The induction of *mer* expression by Hg (II) binding to merR occurs at nanomolar concentration of Hg (II) (Condee and Summers, 1992). Organomercurials induce *mer* operon expression after their cleavage by merB to Hg (II). But, in some cases, operon expression could be induced by organomercurials themselves before cleavages (Nucifora et al., 1989; Kiyono et al., 1997, 2000). Without the induction by low concentrations of Hg (II) (Lund et al., 1986), the "mercury resistance" is not present and cells are toxified at otherwise non-toxic Hg (II) concentrations (Horn et al., 1994). Organomercurial lyases differ in their substrate specificity; some lyases cleave mercury-carbon bonds of only a small number of organomercurials, while many others can cleave many bonds (Nakamura et al., 1990). Bacterial strains examined from many distinct environments, reveal that *mer* operons occur on plasmids (Brown et al., 1986; Griffin et al., 1987; Radstrom et al., 1994) as well as on chromosomes (Wang et al., 1987; Inoue et al., 1991) and, also, are often components of transposons (Misra et al., 1984; Kholodii et al., 1993).

Further, sequence analyses revealed that *mer* operon is a genetic mosaic with a novel modular arrangement (Summers, 1986) of essential genes and accessory genes. Although the presence of mercuric reductase is essential for enzymatic detoxification - and hence resistance to inorganic mercury, expression of *mer*A gene and *mer*R gene has been reported in a high proportion of gram-positive environmental strains sensitive to mercury, suggesting the presence of non-functional *mer* operons in which the mercury transport genes are either absent or non-functional (Bogdanova et al., 1992). Although the physical arrangement of the *mer* operons may vary, all contain the essential genes but surprisingly, only limited studies have attempted to characterize mercury resistance at the molecular level in marine bacterial isolates (Barkay et al., 1989; Rasmussen and Sorensen, 1998). They found that only 12% of culturable MRB from estuarine environments hybridized to *mer* (Tn*21*) probe suggesting that such MRB from the marine environment encode novel *mer* genes or other mechanism (s) that provide Hg resistance (Reyes et al., 1999). It has been proposed that *mer* is an ancient system, which evolved at

a time when levels of available mercury kept rising in natural environments, possibly as a consequence of increased volcanic activity (Osborn et al., 1997).In this thesis mercury resistant bacteria from waste water effluents have been investigated for the *mer* genes from genomic DNA .

5. MATERIAL AND METHODS

5.1. Sample collection (Description of water samples and determination of bacterial load).The sampling area was the industrial waste water effluents from waste water treatment plant of Rourkela Steel Plant (Fig 6, 7, 8a - d). Samples were collected in sterile plastic bottles.

Figure 6. Rourkela Steel Plant

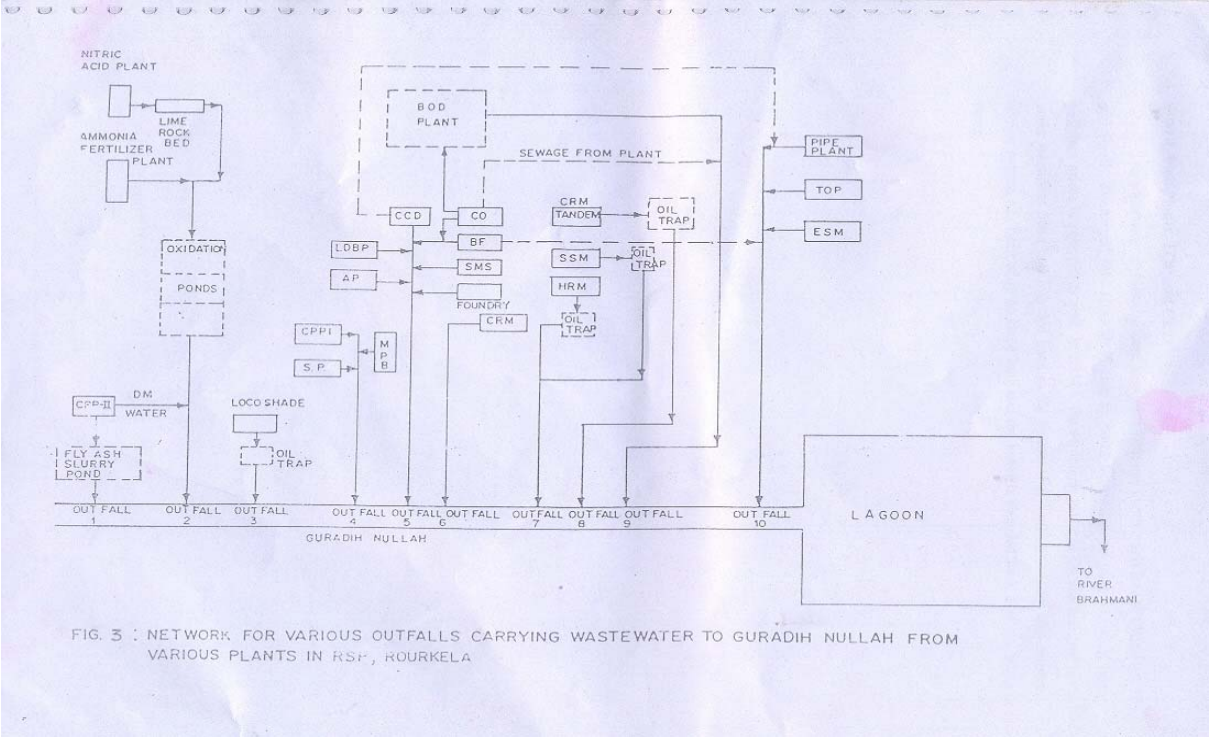


FIG. 3 : NETWORK FOR VARIOUS OUTFALLS CARRYING WASTEWATER TO GURADIH NULLAH FROM VARIOUS PLANTS IN RSP, ROURKELA

Figure 7. Network for various outfalls carrying waste water to Gundah Nullah from various plants in RSP, Rourkela.

(a)

(b)

(c)

(d)

Figure 8 (a to d). Sampling Sites

5.2. Bacterial Enumeration Water samples were serially diluted from 10^{-1} to 10^{-5}, and aliquots each of 0.1ml dilution were inoculated in duplicate plates of Luria Bertani Agar media and Luria Bertani Agar (LBA) media (Composition: 10 g peptone, 5 g yeast extract, 10 g NaCl, 15g agar, 1000 ml Distilled water at pH 7.4) supplemented with 10 ppm of Mercury ($HgCl_2$). All plates were incubated at 37^0C in the incubator for 24hours.Water samples were collected from the lagoon inlet of waste water treatment plant of RSP and were plated on to Luria Bertani agar medium and Luria Bertani Agar medium supplemented with 5 ppm of Hg. Upon noticing very high counts for the first time on all plates with 5 ppm Hg concentration of Hg was increased to 10ppm. We decided to use 10 ppm for MRB enumeration reported here. Plates were incubated at room temperature (37±2°C) and final counts of CFU taken after 24 hrs.C.F.U was calculated as;

$$\text{CFU/ml of original sample} = \frac{\text{No. of Colonies}}{\text{Inoculum size (ml) x Dilution Factor}}$$

The percentage of Mercury resistant bacteria (MRB) was calculated by using the formula

Percentage (%) of mercury resistant bacteria (MRB) = Mecury resistant bacteria x 100/Total Heterotrophic bacteria.

Total plate counts from each sample were also enumerated by plating them on LA without added Hg.After the incubation different cultures were seen on the petri plates. Counting was done for each plate.Isolation of MRB was done by directly plating them on LBA containing mercury($HgCl_2$).In direct method after enumerating the number of bacteria the appeared colonies on appropriate plates which were furhet incubated for 24 hours.The pue cultures of the isolated strains were preserved in LBA medium supplemented with 10 ppm $HgCl_2$ slants in vials under refrigerated conditions (4^oc) and coded as RSP_{11} to RSP_{18} from Site- 1 ; RSP_{2A} to RSP_{2G} from Site- 2 and RSP_{3I} to RSP_{3T} from Site -3 The strains were identified on the basis of their colour and growth. Three microorganisms were selected for further pure culture. Pure culture was obtained by streak plate method. The strains were RSP_{15}, RSP_{2A}, RSP_{2E}, RSP_{3I}.

5.3. Minimum Inhibitory Concetration Test

The above selection was done on the basis of Minimum Inhibitory Concentration of mercury ($HgCl_2$) which they can resist.These four strains showed highest resistant to mercury($HgCl_2$) phenotypically.Minimum Inhibitory Concentration testwas carried out by taking LBA supplemented with 200ppm,100ppm,50ppm,25ppm,12.5ppm Mercury ($HgCl_2$). Two wells were made on each plate by a gel borer .For each strain three plates were used and six wells were made in total .They were filled with 1ml of 200ppm,100ppm,50ppm,25ppm,12.5ppm Mercury ($HgCl_2$) respectively and a control i.e distilled water was also taken. The plates were incubated at 35 ± 2^{o}c for 24 hrs and the zone of inhibition was recored to decipher the minimum concentration of mercury at which the growth of bacteria gets inhibited.

5.4. Antibiotic Susceptibility Test (Hi Media Laboratories)

Then antibiotic susceptibility test was done on them by 9 different antibiotic discs of 30 mcg each (Amoxicillin, Vancomycin, Kanamycin, Ciprofloxacin, Chloramphenicol, Tetracycline, Amikacin, Neomycin, Cefaclor).

- Mueller Hinton Agar plates were prepared for testing the antibiotic susceptibility of the isolated strains.
- Pure cultures were used as inoculums.The colonies were transferred to Lueria Bertani broth (5 ml), incubated at 35 ± 2^{o}C till the development of moderate turbidity.
- A sterile non toxic cotton swab is dipped into the inoculum in the broth and rotated firmly against the upper inside wall of the tube to express excess fluid.The entire agar surface was streaked with the swab for 3 times turning the plate at 60^o angle between each streaking.The inoculums was allowed to dry for 5 mins.
- The discs were dispensed using aseptic technique at least 24 mm apart.
- Petriplates were incubated immediately at 37^{o}c and examined after 16 – 18 hours.The zones showing complete inhibition were measured and the diameter of the zones were measured to the nearest millimeter.

- By the antibiotic zone scale, the area of inhibition was measured for each antibiotic.Sensitivity of the isolates to each antibiotic was determined according to the chart provided by the manufacturer (Himedia, Mumbai).

  They were taken for further biochemical tests. Broth culture was prepared in the nutrient broth. Biochemical test was performed by using the 1 day long inoculated broth culture. Results were interpreted. Triple sugar iron agar test was done by preparing slant culture in test tube and incubation was done by stab and streak culture method. Motility test was performed using Mannitol Motility Agar medium by preparing slant culture in tubes. Aerobic nature of organisms was checked by using Basal Medium. The entire Biochemical tests were analyzed and interpreted.

5.5. Biochemical tests

A] GRAM STAINING

The most important differential stain used in bacteriology is the Gram stain, named after Dr. Christian Gram. It divides bacterial cells into two major groups, gram-positive and gram-negative, which makes it an essential tool for classification and differentiation of microorganisms. The Gram stain reaction is based on the difference in the chemical composition of bacterial cell walls. Gram-positive cells have a thick peptidoglycan layer Whereas the peptidoglycan layer in gram negative cells is much thinner and surrounded by outer lipid-containing layers. Peptidoglycan is mainly a polysaccharide composed of two chemical subunits found only in the bacterial cell wall. These subunits are N-acetylglucosamine and N- acetylmuramic acid. As adjacent layers of peptidoglycan are formed, they are cross linked by short chains of peptides by means of a transpeptidase enzyme, resulting in the shape and rigidity of the cell wall. Early experiments have shown that if the gram positive cell is denuded of its cell wall by the action of lysozyme or penicillin, the gram-positive cell will stain gram negative.

Primary stain

Crystal violet is used first and stains all cells purple. Its function is to impart its color to all cells. In order to establish a colour contrast,

Mordant

Gram's iodine, this reagent is not only a killing agent, but also serves as a mordant a substance that increases the cells' affinity for a stain. It does this by binding to the primary stain, thus forming an insoluble complex. The resultant crystal-violet-iodine complex serves to intensify the colour of the stain. At this point, all cells will appear purple black.

Decolorizing agent

Ethyl alcohol, 95%- This reagent serves as a dual function as a protein-dehydrating agent and as a lipid solvent. Its action is determined by two factors, the concentration of lipids and the thickness of peptidoglycan layer in bacterial cell walls. In gram negative cells, the alcohol increases the porosity of the cell wall by dissolving the lipid in the outer layers. Thus the CV-1 complex can be more easily removed from the thinner and less highly cross linked Peptidoglycan layer. Therefore, the washing out effect of the alcohol facilitates the release of the unbound CV-1 complex, leaving the call colorless or unstained. The much thicker peptidoglycan layer in gram positive cells is responsible for the more stringent retention of the CV-1 complex, as the pores are made smaller due to the dehydrating effect of the alcohol. Thus the tightly bound primary stain complex is difficult to remove, and the cells remain purple.

Counterstain

Safranin is used to stain red those cells that have been previously decolorized. Since only gram negative cells undergo decolourization, they may now absorb the counter stain. Gram positive cells retain the purple colour of the primary stain.

Procedure

- One clean glass slide was taken.
- A smear was prepared by placing a drop of water on the slide and then transferring microorganism to the drop of water with a sterile cooled loop. It was mixed and spread by means a circular motion of the inoculating loop.
- Smear was air dried and heat fixed.
- Smear was gently flooded with crystal violet for 1min.
- Gently washed with tap water.

- Smear was gently flooded with Gram's iodine and left for 1 min.
- Gently washed with tap water.
- Decolorized with 95% ethyl alcohol reagent. It was added drop by drop until no further violet colour comes out.
- Gently washed with tap water.
- Counterstained with safranin for 45 seconds.
- Gently washed with tap water.
- It was dried with bibulous paper and examined under oil immersion.

B] CARBOHYDRATE UTILIZATION TEST (Hi Carbohydrate Kit)

Hi Carbohydrate Kit is a standardized colorimetric identification system utilizing thirty five carbohydrate utilization tests. Tests are based on the principle of pH change and substrate utilization.

a) Carbohydrate fermentation test

• Colour of the medium changes from red colour to yellow colour due to acid production, if the test is positive.

b) ONPG test

• Medium changes from colorless to yellow is the positive test.

• Detects β-galactosidase activity.

c) Esculin hydrolysis

• Colour of the medium changes from cream to black if the test is positive.

d) Citrate utilization

• Colour of the medium changes from yellowish green to blue if the test is positive.

• Detects capability of organism to utilize citrateas as a sole carbon source.

e) Malonate utilization

• Colour of the medium changes from light green to blue if the test is positive.

• Detects capability of organism to utilize malonate as a sole carbon source.

f) Indole test

• 1-2 drop of kovac's reagent was added.

• Development of reddish pink colour within 10sec indicates positive reaction.

g) Nitrate reduction test

•1- 2 drops of sulphanilic acid and 1-2 drops of N, N-Dimethyl-1-Napthylamine reagent were added.

•Immediate development of pinkish red colour on addition of reagent indicates positive reaction.

Preparation of inoculum

1) The organisms to be identified were first isolated and purified. Only pure cultures were used.

2) Kit was opened aseptically aseptically. Each well was inoculated with 50µl of the prepared inoculm by surface inoculation method.

3) The kit can also be inoculated by stabbing each individual well with a loopful of inoculum.

INCUBATION

•Temperature of incubation 35±2^{o}c. Duration of incubation 18-24 hours.

C] CARBOHYDRATE FERMENTATION

Many organism use carbohydrates differently to obtain energy depending on their enzyme complement. Some organisms are capable of fermenting sugars such as glucose anaerobically while others use the aerobic pathway. The purpose of this test is to determine whether an organism can ferment a specific carbohydrate with production of acid.

a) Triple sugar Iron (TSI) and Hydrogen sulfide production (H_2S):

Looks at fermentation of glucose, lactose, and sucrose and checks if hydrogen sulfide is produced in the process. Basically a pH indicator will change the color of the media in response to fermentation…where that color change occurs in the tube will indicate what sugar or sugars were fermented. The presence of a black color indicates that H_2S was produced. In this media, H_2S reacts with the ferrous sulfate in the media to make ferrous sulphide which is black in colour.To inoculate, use a needle to stab agar and then uses a loop to streak the top slated region. In addition to TSI media, SIM media can be used to determine if H_2S is produced. A black color in the SIM medium following inoculation and incubation indicates that H_2S is made by the bacteria.

D] OXIDASE TEST:

To perform this test simply the test culture was made as a smear onto an oxidase dry slide. If a color change to purple or blue is evident at 30 seconds in1 minute then the result is positive. It is important that the test is read by one minute to ensure accurate results (avoid false negatives and false positives). This laboratory test is based on detecting the production of the enzyme cytochrome oxidase by Gram-negative bacteria. It is a hallmark test for the *Neiserria*. It is also used to discriminate between aerobic Gram-negative organisms like *Pseudomonas aeruginosa* and other *Enterobacteriaciae*.

E] MOTILITY TEST:

The motility test is not a biochemical test since we are not looking at metabolic properties of the bacteria. Rather, this test can be used to check for the ability of bacteria to migrate away from a line of inoculation thanks to physical features like flagella. To perform this test, the bacterial sample is inoculated into mannitol motility agar media (Composition - Peptone 20.0 g,Mannitol 2.0 g,Potassium Nitrate 1.0 g,Phenol Red 0.04 g and Agar 5.0 g per litre maintained at a pH 7.3 ± 0.2)using a needle. Simply stab the media in as straight a line as possible and withdraw the needle very carefully to avoid destroying the straight line. After incubating the sample for 24-48 hours observations can be made. Check to see if the bacteria have migrated away from the original line of inoculation. If migration away from the line of inoculation is evident then you can conclude that the test organism is motile (positive test). Lack of migration away from the line of inoculation indicates a lack of motility (negative test result).

F] DETECTION OF ENDOSPORES :-

The 24 hour bacterial culture was kept in a hot air oven maintained at 80 degrees Celsius to kill the cells. The test tubes were incubated at 37 ^{o}c for 1 day .Turbidity indicated the formation of endospores.

6. RESULTS

6.1. Enumeration of MRB :- Total Heterotrophic bacterial population (THB) ranged from 0.213 x 10^5 ±0.004 to 3.9 x 10^5 for Site – 1, 0.2185 x 10^5 ± 0.009 to 4.8 x 10^5 ± 0.141 for Site – 2 and 0.208 x 10^5 ±0.011 to 1.0 x 10^5 ±0.028 for Site – 3.Mercury resistant bacterial Population (MRB) ranged from 0.1455 x 10^5 ±0.014 to 5 x 10^5 ±2.828 for Site -1, 0.164 x 10^5 ±0.033 to 8 x 10^5 ±4.242 for Site – 2 and 0.14 x 10^5 ± 0.024 to 0.4 x 10^5 ± 0.141for Site- 3.Percentage of mercury resistance varied between 14.3±3.252 to 72.55±2.333 for Site – 1 , 23.7±14..990 to 80±4.949 for Site – 2 and 24.5±4.101 to 67.7±15.273 for Site – 3.Highest number of MRB were obtained from Site -2 (Table – 1).

Table 1 :- ENUMERATION OF THB & MRB

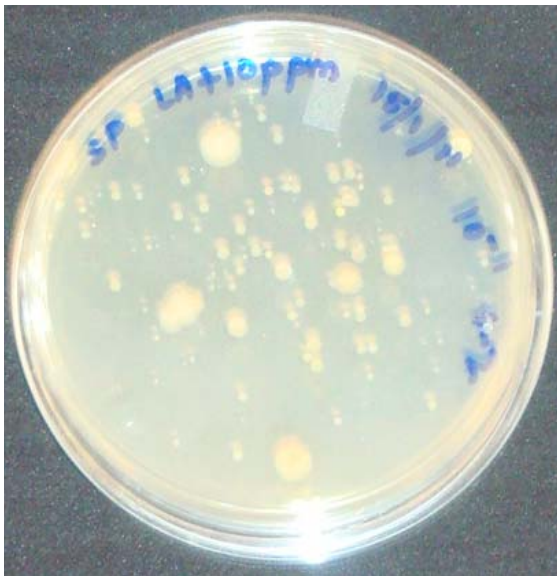
Site	Sample	Hg Concentration	Dilutions	THB x10^5(CFU/ml)±SD	MRB x 10^5(CFU/ml) ±SD	% of Hg resistance
1	Water	10 ppm	10^{-1}	0.213 ±0.004	0.1455±0.014	72.55±2.333
		10 ppm	10^{-2}	1.08 ±0.0282	0.415±0.516	54.8±10.889
		10 ppm	10^{-3}	3.9 ±0	1.25±0.353	32±9.050
		10 ppm	10^{-4}	20 ±7.07	5±2.828	14.3±3.252
		10 ppm	10^{-5}	-	-	-

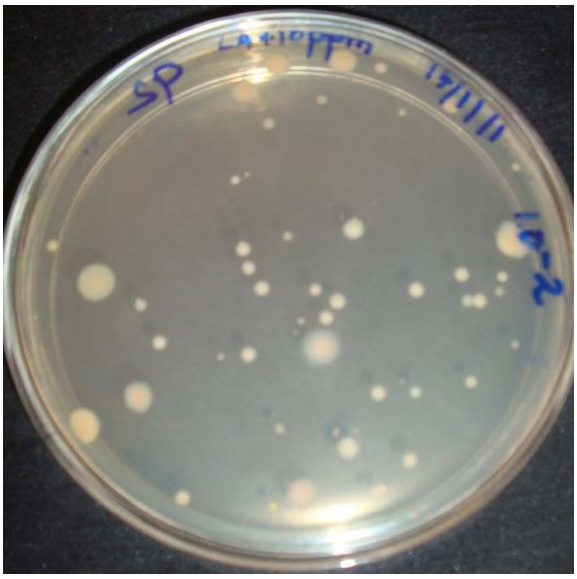
Sites	Sample	Hg Concentration	Dilutions	THB x10^5(CFU/ml) ±SD	MRB x 10^5(CFU/ml) ±SD	% of Hg resistance
2	Water	10 ppm	10^{-1}	0.2185±0.009	0.164±0.033	80±4.949
		10 ppm	10^{-2}	1.485±0.035	0.895±0.190	70.5±1.909
		10 ppm	10^{-3}	4.8±0.141	3.65±0.212	55.4±4.808
		10 ppm	10^{-4}	35±4.242	8±4.242	23.7±14..990
		10 ppm	10^{-5}	-	-	-

Sites	Sample	Hg Concentration	Dilutions	THB x10^5(CFU/ml) ±SD	MRB x 10^5(CFU/ml) ±SD	% of Hg resistance
3	Water	10 ppm	10^{-1}	0.208±0.011	0.14±0.024	67.7±15.273
		10 ppm	10^{-2}	0.5±0.421	0.32±0.042	38±0.848
		10 ppm	10^{-3}	1.0±0.028	0.4±0.141	24.5±4.101
		10 ppm	10^{-4}	-	-	-
		10 ppm	10^{-5}	-	-	-

Plate –I

Isolated Bacterial Colonies from Site - 1





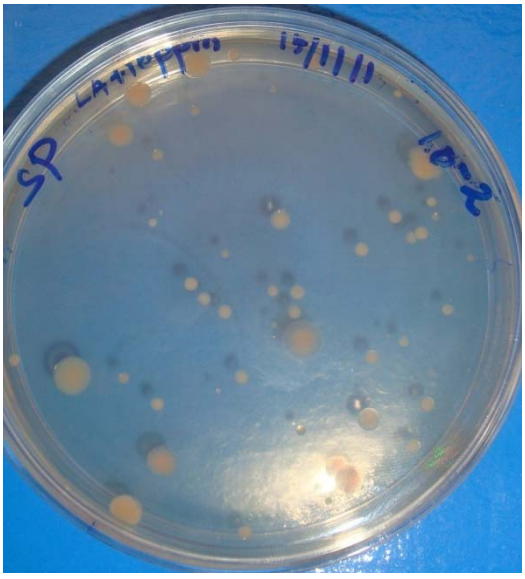
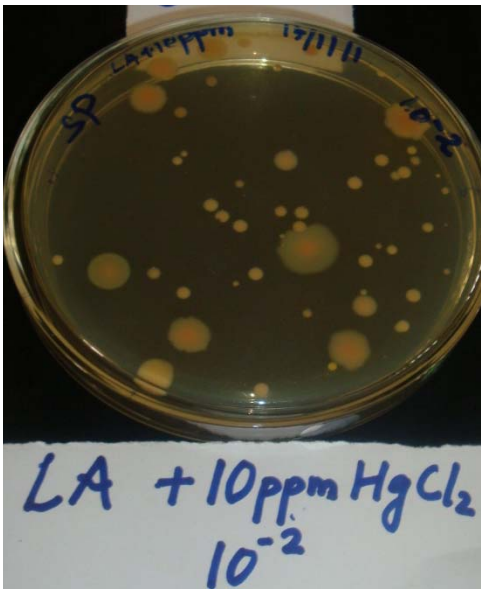


Plate –II

Isolated Bacterial Colonies from Site – 2



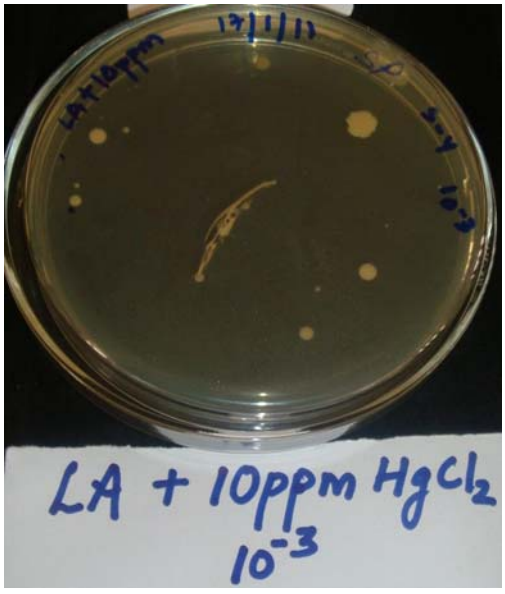
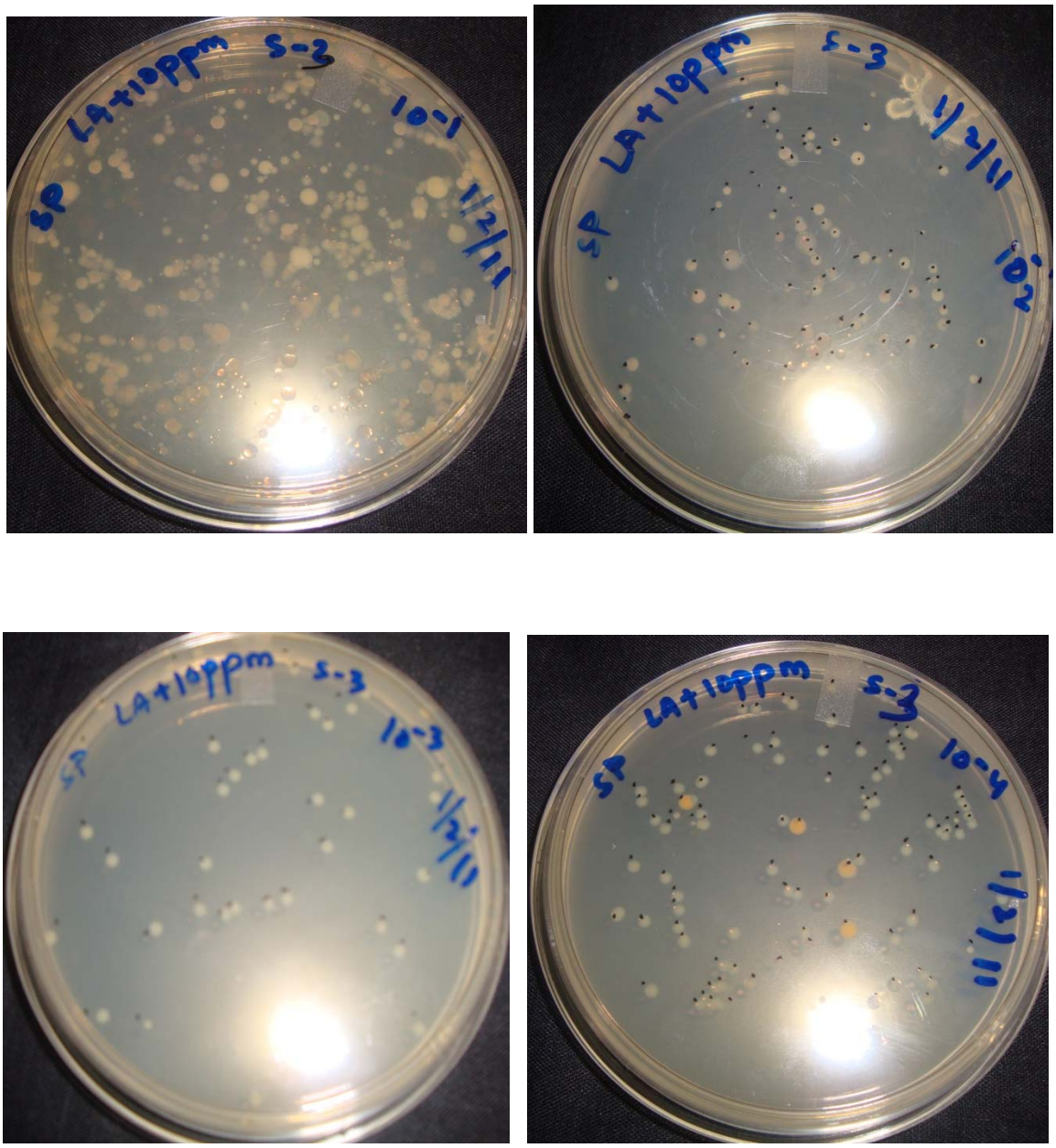


Plate –III

Isolated Bacterial Colonies from Site – 3



6.2. Characteristics of isolated colonies

The isolated bacterial colonies were either yellowish,whitish or orange coloured.They were small,elongated and circular in shape. Most of them were found to have an entire margin, were medium sized and flat.They were slimy in appearance (Table 2).

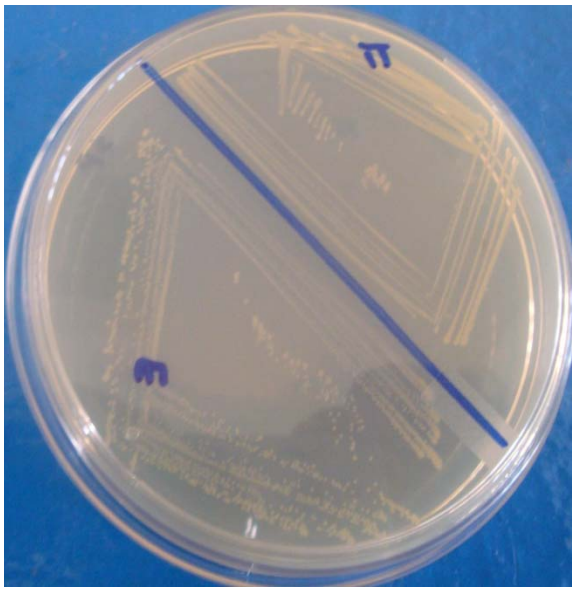
Table 2:- CHARACTERISTICS OF ISOLATED COLONIES

STRAINS	**Isolated Colony Morphology**
RSP_{11}	Milky white, translucent, slimy, medium sized, rounded, entire.
RSP_{12}	Lemon Yellow, small, rounded,entire,opaque.
RSP_{13}	White,circular ,opaque,medium sized.
RSP_{14}	Yellow,small,rounded,entire margin.
RSP_{15}	Creamy(off white),long ,elevated,opaque.
RSP_{16}	Yellow , small , rounded,entire,opaque.
RSP_{17}	Yellow,small,rounded,entire margin.
RSP_{18}	White,opaque,middle sized,entire margin.
RSP_{2A}	Orangish cream, small colonies, long, opaque, entire.
RSP_{2B}	White, transluscent, bulged, large sized, irregular colonies.
RSP_{2C}	White, transluscent, slimy, medium sized, rounded, entire.
RSP_{2D}	Yellow, small , rounded,entire,opaque.
RSP_{2E}	Dusty white,medium sized,opaque.
RSP_{2F}	Offwhite,Circular,entire margin ,
RSP_{2G}	Pinkish,small colonies,circular,opaque
RSP_{2H}	Yellow,small,rounded,entire margin.
RSP_{3I}	Orange, small colonies,long,opaque,entire.

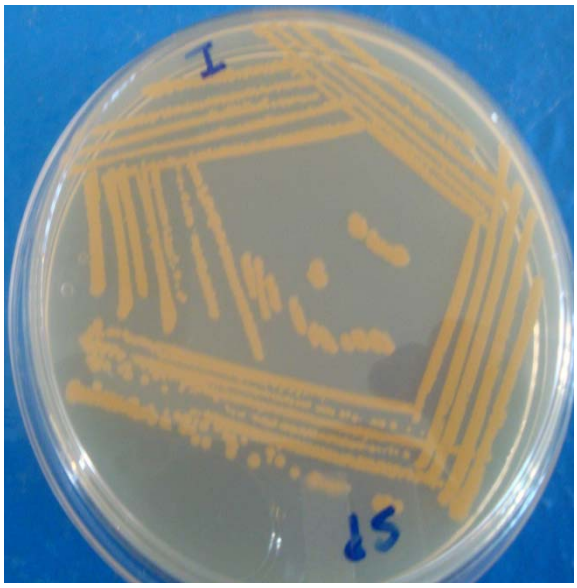
RSP_{3J}	Milky white,circular,opaque,medium sized.
RSP_{3K}	Small,offwhite,Circular,entire margin , Flat
RSP_{3L}	Yellow,small,rounded,entire margin.
RSP_{3M}	White,circular ,opaque,medium sized.
RSP_{3N}	White,Circular,entire margin ,
RSP_{3O}	White, circular ,opaque,medium sized.
RSP_{3P}	Yellow, small , rounded,entire,opaque.
RSP_{3Q}	White,opaque,middle sized,entire margin.
RSP_{3R}	Yellow,small,rounded,entire margin.
RSP_{3S}	Yellow,Small,rounded,entire margin.
RSP_{3T}	Yellow,Medium,circular,Entire margin.

PLATE IV

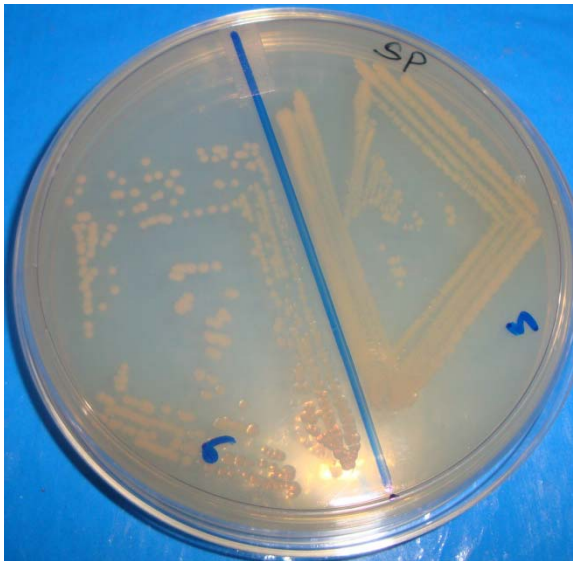
Pure Culture of bacterial strains



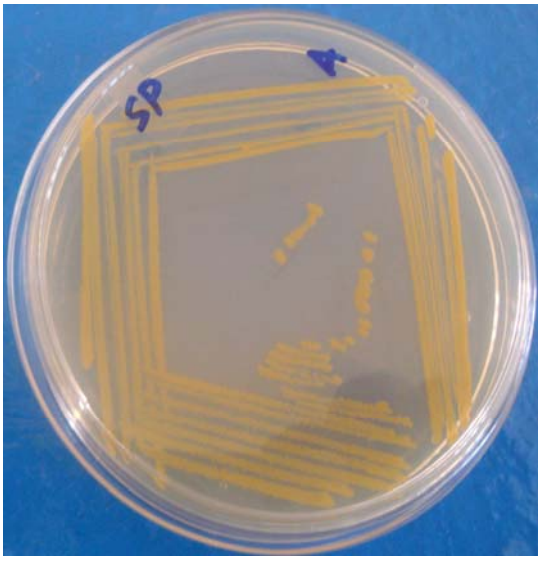
(STRAIN – RSP_{2E})



(STRAIN – RSP_{3I})



(STRAIN – RSP_{15})



(STRAIN – RSP_{2A})

6.3. Minimum inhibitory concentration (MIC)

Minimum Inhibitory Concentration refers to the minimum concentration of mercury at which bacterial growth can be inhibited. After the MIC test it was concluded that out of the twenty eight isolates from 3 different sites the strains namely **RSP_{15} , RSP_{2A} , RSP_{2E}** and **RSP_{3I}** were inhibited when grown on LBA media supplemented with 25 ppm of $HgCl_2$. They showed a zone of inhibition of 9 mm , 8mm , 6mm and 6 mm respectively (Table – 3).

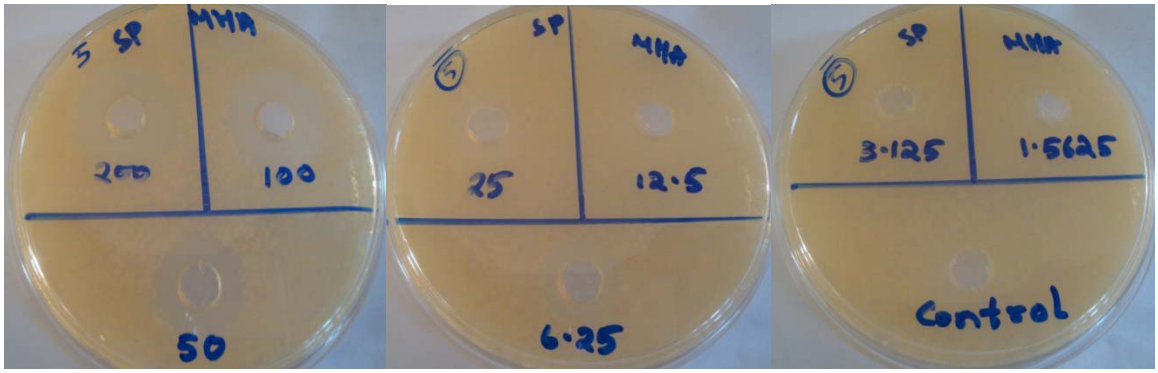
Table 3. MINIMUM INHIBITORY CONCENTRATION (MIC)

STRAINS	200 ppm	100 ppm	50 ppm	25 ppm	12.5 ppm	6.25 ppm	3.125 ppm	1.5625 ppm	CONTROL (Distilled Water)
RSP_{11}	20	18	16	14	-	-	-	-	-
RSP_{12}	22	19	12	12	-	-	-	-	-
RSP_{13}	27	25	22	19	-	-	-	-	-
RSP_{14}	28	24	22	19	-	-	-	-	-
RSP_{15}	**22**	**18**	**14**	**9**	**-**	**-**	**-**	**-**	**-**
RSP_{16}	28	23	21	18	-	-	-	-	-
RSP_{17}	23	20	19	17	-	-	-	-	-
RSP_{18}	24	20	18	15	-	-	-	-	-
RSP_{2A}	**20**	**17**	**12**	**8**	**-**	**-**	**-**	**-**	**-**
RSP_{2B}	20	18	16	14	-	-	-	-	-
RSP_{2C}	25	22	20	18	-	-	-	-	-
RSP_{2D}	28	26	24	22	-	-	-	-	-
RSP_{2E}	**16**	**12**	**10**	**6**	**-**	**-**	**-**	**-**	**-**
RSP_{2F}	26	22	20	16	-	-	-	-	-
RSP_{2G}	24	22	18	14	-	-	-	-	-
RSP_{2H}	20	18	15	13	-	-	-	-	-
RSP_{3I}	**16**	**12**	**8**	**6**	**-**	**-**	**-**	**-**	**-**
RSP_{3J}	28	26	23	21	-	-	-	-	-
RSP_{3K}	20	18	16	14	-	-	-	-	-
RSP_{3L}	20	17	14	13	-	-	-	-	-
RSP_{3M}	22	20	19	15	-	-	-	-	-
RSP_{3N}	22	19	16	14	-	-	-	-	-

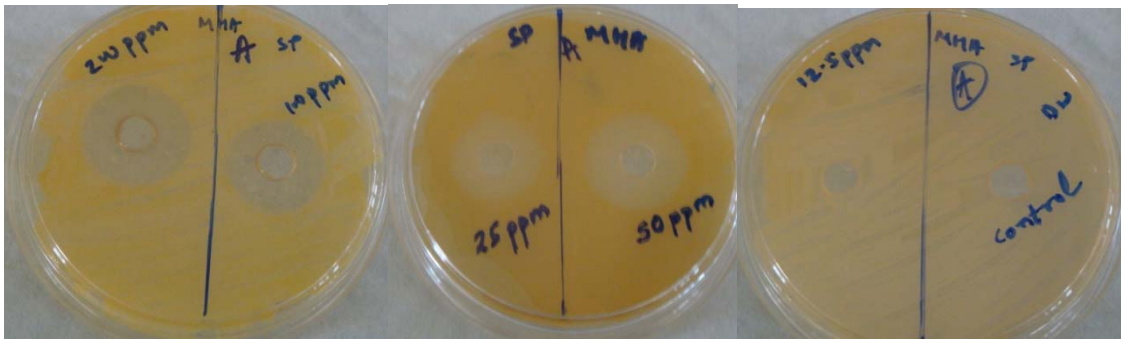
RSP_{3O}	22	19	18	14	-	-	-	-	-
RSP_{3P}	24	21	19	16	-	-	-	-	-
RSP_{3Q}	21	20	19	16	-	-	-	-	-
RSP_{3R}	24	22	18	16	-	-	-	-	-
RSP_{3S}	20	18	16	12	-	-	-	-	-
RSP_{3T}	22	20	19	15	-	-	-	-	-

PLATE V

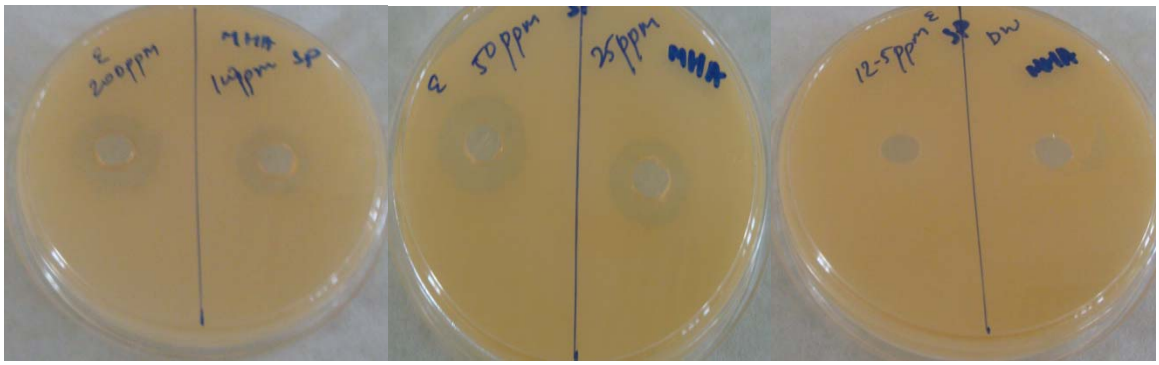
Minimum inhibitory concentration



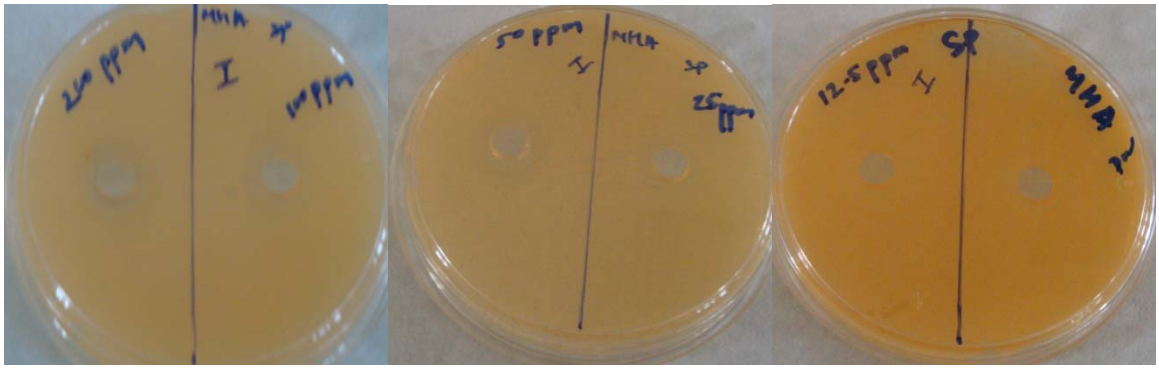
(STRAIN – RSP_{15})



(STRAIN – RSP_{2A})



(STRAIN – RSP_{2E})



(STRAIN – RSP_{3I})

6.4. Morphology of isolated strains -

All the four strains isolated were rod shaped, were Gram negative, aerobic, produced endospores and 3 among them were motile.Out of all the strains isolated only RSP_{3I} could effectively utilize glucose (Table 4).

Table 4 :- Colony morphology, cell morphology, Gram stain reaction, and general properties of streaked bacterial species :-

Strains	Cell Morphology				Gram's Test	O_2 Use	Glucose Use	Endospores (Y/N)	Motility (Y/N)
	Shape	Elevation	Colour	Margin					
RSP_{15}	Rod	Yes	Creamy White	Entire	-Ve	Yes	No	Y	N
RSP_{2A}	Rod	Yes	Orangish Cream	Entire	-Ve	Yes	No	Y	Y
RSP_{2E}	Rod	Yes	White	Entire	-Ve	Yes	No	Y	Y
RSP_{3I}	Rod	Yes	Orange	Entire	-Ve	Yes	Yes	Y	Y

6.5. Antibiotic Susceptibility Test

By performing Antibiotic Susceptiblity Test it was concluded that the strain RSP_{2E} was resistant to all most all antibiotics except Chloramphenical (Table – 5).

Table 5:- Antibiotic Susceptibility Test:-

1. **RSP_{15}**

Sl.no	ANTIBIOTIC	RADIUS-mm	AREA-mm^2
1.	Amoxicillin	20	1257.1429
2.	Vancomycin	-	-
3.	Kanamycin	35	3850
4.	Ciprofloxacin	25	1964.2857
5.	Chloramphenicol	45	6364.2857
6.	Tetracycline	26	2124.5714
7.	Amikacin	36	4073.1429
8.	Neomycin	36	4073.1429
9.	Cefaclor	41	5283.1429

2. **RSP_{2A}**

Sl.no	ANTIBIOTIC	RADIUS-mm	AREA-mm^2
1.	Amoxicillin	30	2828.5714
2.	Vancomycin	20	1257.1429
3.	Kanamycin	23	1662.5714
4.	Ciprofloxacin	26	2124.5714
5.	Chloramphenicol	40	5028.5714
6.	Tetracycline	25	1964.2857
7.	Amikacin	36	4073.1429
8.	Neomycin	36	4073.1429

9.	Cefaclor	41	5283.1429

3. **RSP $_{2E}$**

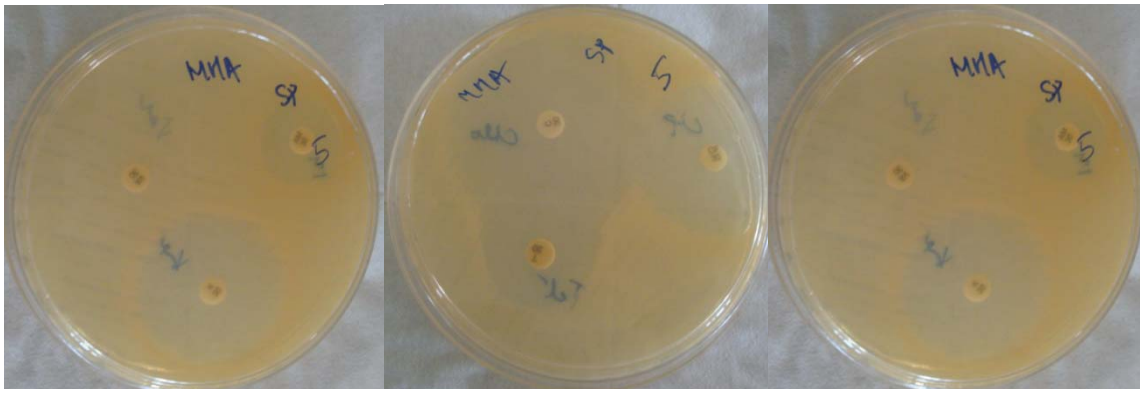
Sl.no	ANTIBIOTIC	RADIUS-mm	AREA-mm^2
1.	Amoxicillin	-	-
2.	Vancomycin	-	-
3.	Kanamycin	-	-
4.	Ciprofloxacin	-	-
5.	Chloramphenicol	22	1521.14286
6.	Tetracycline	-	-
7.	Amikacin	-	-
8.	Neomycin	-	-
9.	Cefaclor	-	-

4. **RSP $_{3I}$**

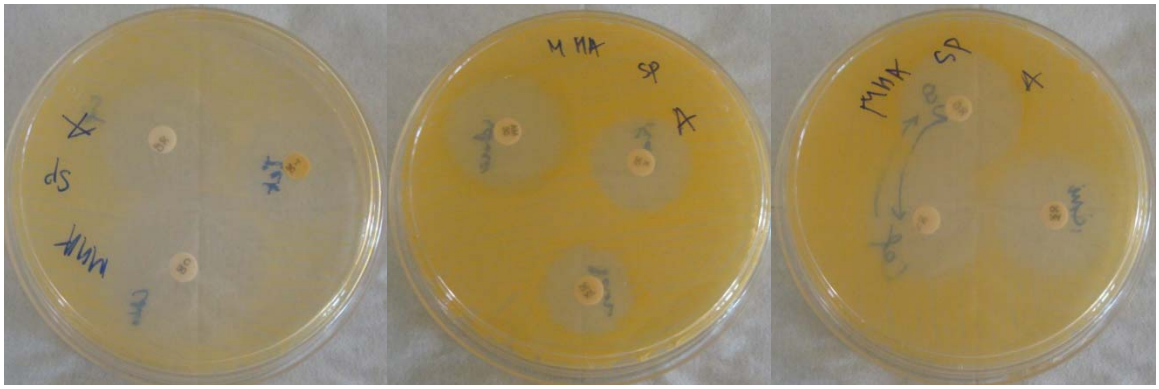
Sl.no	ANTIBIOTIC	RADIUS-mm	AREA-mm^2
1.	Amoxicillin	30	2828.5714
2.	Vancomycin	15	565.71429
3.	Kanamycin	20	1257.1429
4.	Ciprofloxacin	26	2124.5714
5.	Chloramphenicol	22	1521.14286
6.	Tetracycline	20	1257.1429
7.	Amikacin	18	1018.2857
8.	Neomycin	25	1964.2857
9.	Cefaclor	31	3020.2857

PLATE VI

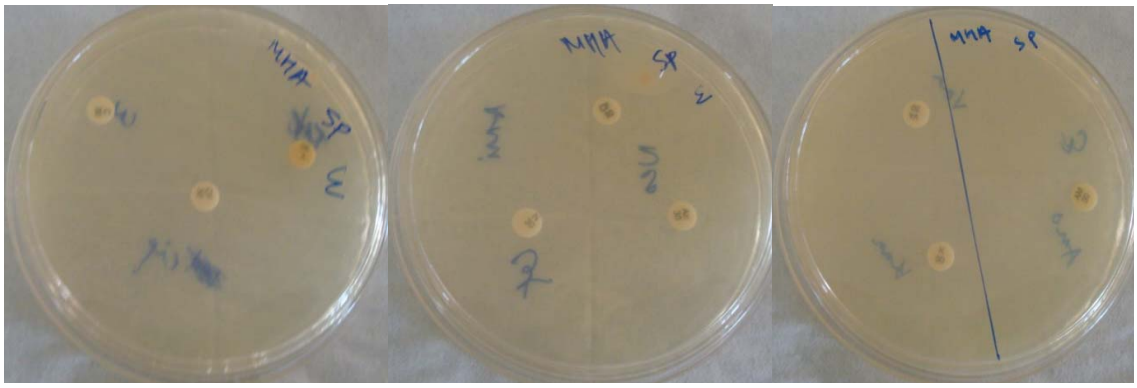
Antibiotic Susceptibility Test



(STRAIN – RSP_{15})



(STRAIN – RSP_{2A})



(STRAIN – RSP_{2E})

(STRAIN – RSP_{3I})

6.6. Biochemical Tests

After performing the biochemical test using the Hicarbo Identification kit , it was decipherd that the strains RSP_{15} , RSP_{2A} , RSP_{2E} showed negative test for carbohydrate utilization unlike RSP_{3I}.(Table 6).

Table - 6 Biochemical Tests :- (HiCarbo Kit- Part A , Part B , Part C)

Sources	RSP_{15}	RSP_{2A}	RSP_{2E}	RSP_{3I}
Lactose	-ve	-ve	-ve	+ve
Xylose	-ve	-ve	-ve	+ve
Maltose	-ve	-ve	-ve	+ve
Fructose	-ve	-ve	-ve	+ve
Dextrose	-ve	-ve	-ve	+ve
Galactose	-ve	-ve	-ve	+ve
Raffinose	-ve	-ve	-ve	+ve
Trehalose	-ve	-ve	+ve	- ve
Melibiose	-ve	-ve	-ve	+ve
Sucrose	-ve	-ve	-ve	+ve
L – Arabinose	-ve	-ve	-ve	+ve
Mannose	-ve	-ve	-ve	+ve
Inulin	-ve	-ve	-ve	+ve
Sodium gluconate	-ve	-ve	-ve	+ve
Glycerol	-ve	-ve	-ve	+ve
Salicin	-ve	-ve	-ve	+ve
Dulcitol	-ve	-ve	-ve	- ve
Inositol	-ve	-ve	-ve	- ve
Sorbitol	-ve	-ve	-ve	- ve
Mannitol	-ve	-ve	-ve	+ve
Adonitol	-ve	-ve	-ve	+ve
Arabitol	-ve	-ve	-ve	- ve
Erythritol	-ve	-ve	-ve	- ve
α methyl D glucoside	-ve	-ve	-ve	- ve
Rhamnose	-ve	-ve	-ve	- ve
Cellobiose	-ve	-ve	-ve	+ ve
Melezitose	-ve	-ve	-ve	- ve
α methyl mannoside	-ve	-ve	-ve	- ve
Xylitol	-ve	-ve	-ve	- ve
ONPG	-ve	-ve	-ve	+ve
Esculin hydrolysis	-ve	-ve	-ve	+ve
D Arabinose	-ve	-ve	-ve	+ve
Citrate Utilization	+ve	+ve	+ve	+ve
Malonate Utilization	+ve	+ve	+ve	+ve
Sorbose	-ve	-ve	-ve	- ve
Control	Pink	Pink	Pink	Pink

6.7. Biochemical Tests

After performing the below mentioned tests using the Enterobacteriaceae Identification kit , it was decipherd that the strains RSP_{15} , RSP_{2A} , RSP_{2E} and RSP_{3I} showed positive test for Lysine,Ornithine,Malonate and Citrate utilization and Nitrate reduction test for.(Table – 7).Oxidase tests for all the strains were positive indicating them to be aerobic strains.(Table 7)

Table – 7 Enterobacteriaceae Identification Kit results

Sources	**RSP_{15}**	**RSP_{2A}**	**RSP_{2E}**	**RSP_{3I}**
ONPG	-ve	-ve	-ve	-ve
Lysine Utilization	+ve	+ve	+ve	+ve
Ornithine Utilization	+ve	+ve	+ve	+ve
Urease	-ve	-ve	-ve	-ve
Phenylalanine deamination	-ve	-ve	-ve	-ve
Nitrate reduction	+ve	+ve	+ve	+ve
H_2S Production	-ve	-ve	-ve	-ve
Citrate Utilization	+ve	+ve	+ve	+ve
Voges Proskauer's	-ve	-ve	-ve	-ve
Methyl Red	-ve	+ve	-ve	-ve
Indole	-ve	-ve	-ve	+ve
Malonate Utilization	+ve	+ve	+ve	+ve
Esculin Hydrolysis	-ve	-ve	-ve	+ve
Arabinose	-ve	-ve	-ve	+ve
Xylose	-ve	-ve	-ve	-ve
Adonitol	-ve	-ve	-ve	-ve
Rhamnose	-ve	-ve	-ve	- ve
Cellobiose	-ve	-ve	-ve	+ve
Melibiose	-ve	-ve	-ve	+ve
Saccharose	-ve	-ve	-ve	+ve
Raffinose	-ve	-ve	-ve	-ve
Trehalose	-ve	-ve	+ve	+ve
Glucose	-ve	-ve	-ve	+ve
Lactose	-ve	-ve	-ve	- ve
Oxidase	+ve	+ve	+ve	+ve

6.8. Triple Sugar Iron Test

The TSI slant is a test tube that contains agar, a pH-sensitive dye (phenol red), 1% lactose, 1% sucrose, 0.1% glucose as well as sodium thiosulfate and ferrous sulfate or ferrous ammonium sulfate.Carbohydrate fermentation is indicated by a yellow colorationof the medium. If the medium in the butt of the tube becomes yellow (acidic), but the medium in the slant becomes red (alkaline), the organism being tested only ferments dextrose (glucose). A yellow (acidic) color in the slant and butt indicates that the organism being tested ferments dextrose, lactose and/orsucrose.A red (alkaline) color in the slant and butt indicates that the organism being tested is a nonfermenter (Table 8).

Table – 8 Triple Sugar Iron Test

Strains	Colour of the butt	Colour of the Slant	Inference
RSP_{15}	Red	Red	Non fermenter
RSP_{2A}	Red	Red	Non fermenter
RSP_{2E}	Red	Red	Non fermenter
RSP_{3I}	Yellow	Yellow	Fermeter

6.9. Mannitol Motility Test

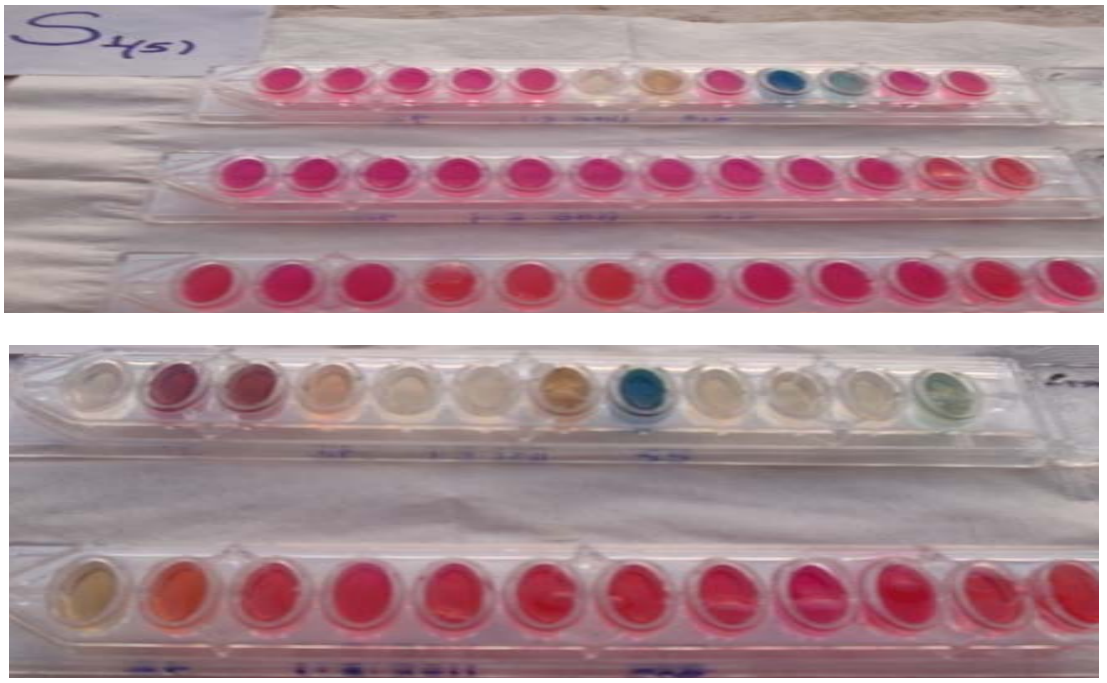
Mannitol motility test agar is a semisolid medium used for the detection of motility Bacterial motility can be observed directly from examination of the tubes following incubation. Growth spreads out from the line of inoculation if the organism is motile. Highly motile organisms provide growth throughout the tube. Growth of non-motile organisms only occurs along the stab line.Generally, if the entire tube is turbid, this indicates that the bacteria have moved away from the stab mark (are motile). The organisms in the three tubes pictured on the right are motile. If, however, the stab mark is clearly visible and the rest of the tube is not turbid, the organism is likely nonmotile (tube pictured on the left).

6.10. Endospore Formation Test

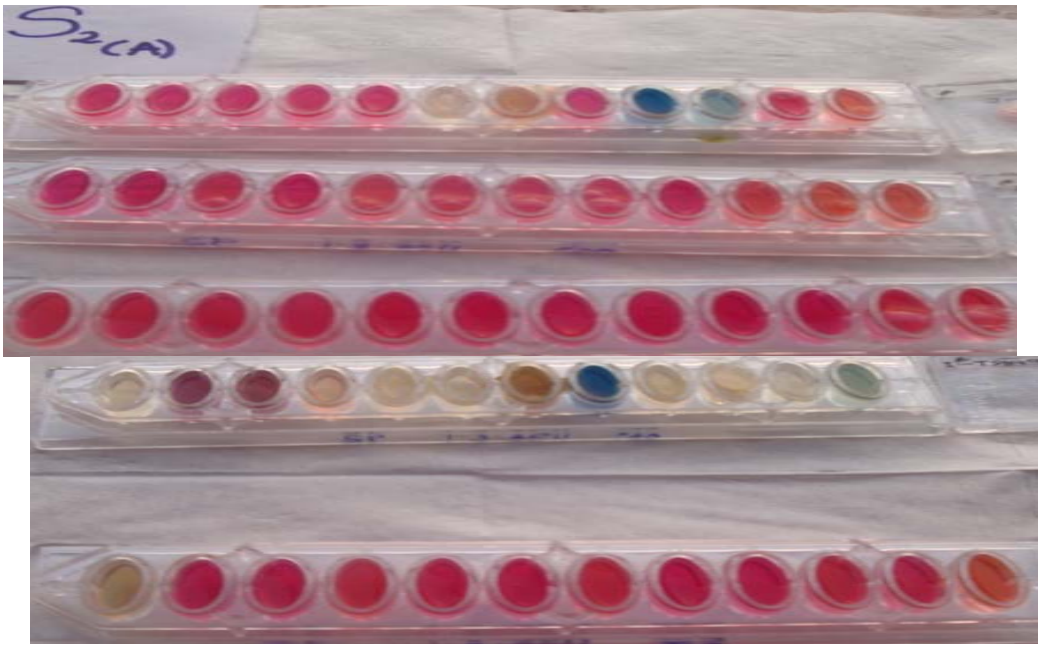
The 24 hour bacterial culture was kept in a hot air oven maintained at 80 degrees Celsius to kill the cells. The test tubes were incubated at 37 ^{o}c for 1 day .Turbidity indicated the formation of endospores.All the strains were endosporous in nature.

PLATE VII

Biochemical Test results



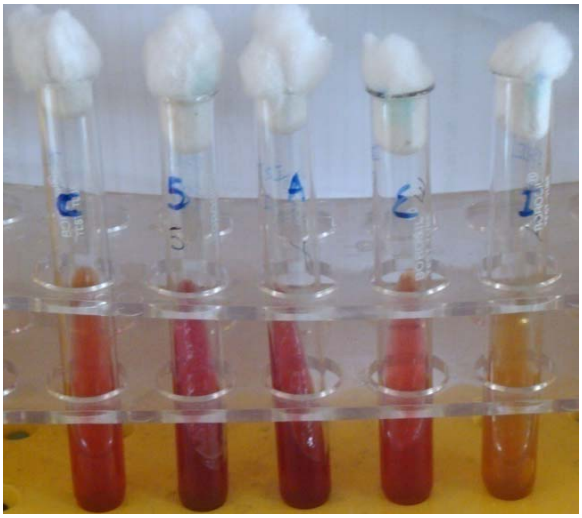
(STRAIN – RSP_{15})



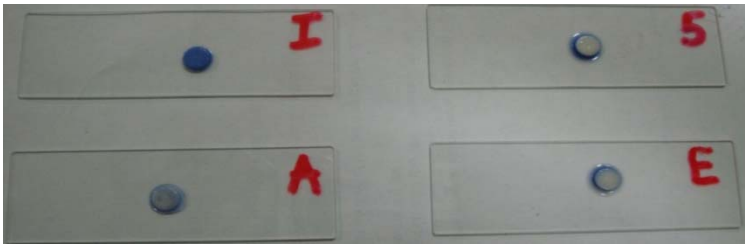
(STRAIN – RSP_{2A})

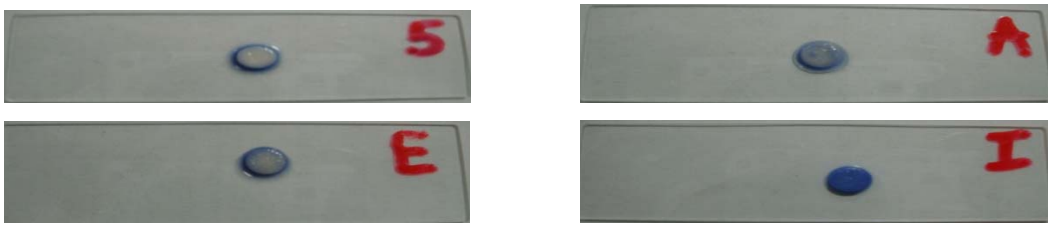
(STRAIN – RSP_{2E})

(STRAIN – RSP_{3I})



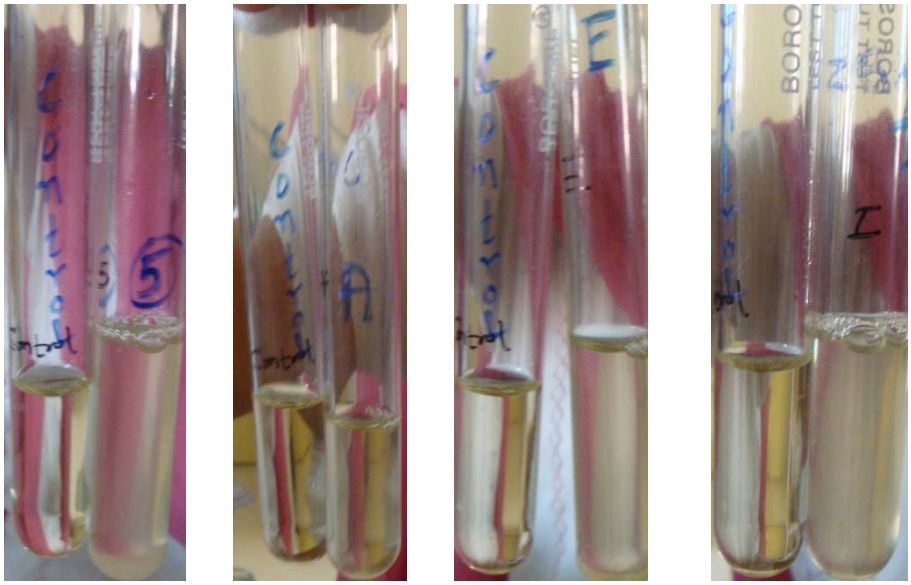
Triple Sugar Iron Agar Test





Oxidase Test

Motility Test



Endospore Formation

7. DISCUSSION

Studies have been carried out to see the Minimum concentration of mercury at which the growth of MRB was inhibited. Enumeration of MRB was carried out from water samples. The water samples from site 1 had MRB varying between 0.15 x 10^5 to 7 x 10^5 CFU/ml , site 2 varying between 0.14 x 10^5 to 5 x 10^5 CFU/ml and site 3 varying between 0.2 x 10^5 to 0.59 x10^5 CFU/ml.Total Heterotrophic bacterial population (THB) ranged from 0.213 x 10^5 ±0.004 to 3.9 x 10^5 for Site – 1, 0.2185 x 10^5 ± 0.009 to 4.8 x 10^5 ± 0.141 for Site – 2 and 0.208 x 10^5 ±0.011 to 1.0 x 10^5 ±0.028 for Site – 3.Mercury resistant bacterial Population (MRB) ranged from 0.1455 x 10^5 ±0.014 to 5 x 10^5 ±2.828 for Site -1, 0.164 x 10^5 ±0.033 to 8 x 10^5 ±4.242 for Site – 2 and 0.14 x 10^5 ± 0.024 to 0.4 x 10^5 ± 0.141for Site- 3.Percentage of mercury resistance varied between 14.3±3.252 to 72.55±2.333 for Site – 1 , 23.7±14..990 to 80±4.949 for Site – 2 and 24.5±4.101 to 67.7±15.273 for Site – 3.Highest number of MRB were obtained from Site -2 Twenty eight randomly isolated environmental strains of these MRB were characterized biochemically. In addition 1 strain of MRB which showed highest resistance to antibiotics and minimum inhibitory concentration was investigated for presence of *mer* operon .

A total of 28 mercury-resistant bacteria from the lagoon inlets of RSP ,Rourkela, India were isolated on selective LA medium amended with 10 ppm mercury. After the MIC test it was concluded that out of the twenty eight isolates from 3 different sites the strains namely **RSP_{15} , RSP_{2A} , RSP_{2E}** and **RSP_{3I}** were inhibited when grown on LBA media supplemented with 25 ppm of $HgCl_2$. They showed a zone of inhibition of 9 mm , 8mm , 6mm and 6 mm respectively.Four of these bacteria were submitted to an extensive biochemical characterization protocols by following MacFaddin (1980). These bacteria were highly resistant to an array of antibiotics conforming to the fact that most of the times heavy metal- resistance is associated with antibiotic resistance. Four of these thirty isolates which tolerated mercury concentration of 25 ppm, termed as bacteria highly resistant to mercury.The amount of mercury in the water sample was estimated to be 4325 ppb by Flameless Atomic Absorption Spectrometry. After several biochemical tests of the four isolated gram negative rods it was deciphered that they belonged to the

pseudomonas strains ; the strain RSP_{15} has got similarity of 99 % with *Comamonas testosterone* and the strain RSP_{2A} has got similarity of 99% with *Pseudomonas marincola*, the strain RSP_{2E} has got similarity of 99% with *Pseudomonas borbori* and the strain RSP_{3I} has got 85 % similarity with *Pseudomonas panacis.* The strain identification was done by online software "ABIS 6 online v. 1.02".

Recent increase in MRB is important considering the present status of mercury consumption in India. Staggering abundances of MRB are useful not only to ascertain the recent changes in the environs of the country have gone through in terms of Hg increases, but also, as a reliable practical method, their enumeration will prove useful, akin to other indicators, the extent of Hg and other heavy metals pollution. This study supports the fact that heavy metalresistance does not arise by chance, rather there has to be a selection pressure from natural and/or anthropogenic inputs to bring this change. The fact that India holds currently the *numero uno*position in the list of mercury-consuming countries, is indicative enough in ascertaining as to why there was an unusual rise in MRB

Corroborating quite well with this observation is the increase in mercury concentration in the environment as reported by Central Pollution Control Board and others. The MRB must be possessing mechanism(s) to deal with not only Hg, but several other toxic pollutants including heavy metals and xenobiotics.

Due to prolonged exposure to mercury and other heavy metal cations/anions, bacteria can acquire highly specific resistance (Barkay, 1987; Rasmussen and Sorensen, 1998, 2001). Some bacteria can reduce Hg^{2+} and most organomercurials to elemental Hg^0, which volatilizes out of the system due to high vapour pressure (Fitzgerald and Mason, 1997). There is a considerable evidence on mercury resistance among common microbial species (Amy & Morita, 1983; Compeau & Bartha, 1984; Colwell et al., 1985; Barkay & Turner, 1997; Barkay, 2000). Some bacteria can convert Hg^{2+} to methyl and dimethyl mercury (Gerlach, 1981). Organomercurial lyase that cleaves the carbon-mercury bonds of certain organomercurials and mercuric reductase that reduces Hg^{2+} to volatile mercury helps bacterial cells in detoxifying mercury compounds (Barkay, 1987). The abilities of environmental isolates to tolerate Hg, its various ionic and molecular

forms are of greater interest in microbial ecology. Many previous studies from the European and North American coasts have reported the occurrence of culturable heterotrophic bacteria capable of tolerating ca. 0.5 ppm Hg from locations affected by a variety of anthropogenic activities (Barkay, 1987; Rasmussen and Sørensen, 1998; Reyes et al., 1999). An increase in the heavy metal resistant fraction of culturable heterotrophic bacteria in the aquatic ecosystems is ascribed to the growth primarily of mercury-resistant bacteria (Barkay and Olson, 1987; Müller et al., 2001b; Rasmussen and Sørensen, 1998, 2001). Similar to the results obtained during this study prior to 1999, none to 10% of the CFU have been reported to be growing in general nutrient agar media amended with 0.5 ppm Hg. Rasmussen and Sørensen (1998) noticed high levels of self-transmissible Hg resistance plasmids in bacterial communities from a mercury-contaminated site inside the Copenhagen Harbor which had higher abundance of MRB.Silver and Phung (1996) proposed that toxic heavy metal resistance determinants might be preexistent to human activities. In all likelihood, varieties of natural prokaryote assemblages possessing resistance mechanisms would get 'selected' as a result of human pollution (Förstner and Wittmann, 1979) in recent centuries. Such assemblages are of continued relevance in ecosystem stability. It is therefore, of pertinence to take note of adaptive responses by native microflora and decipher their involvement in biogeochemical cycling of mercury on a global scale. Heavy metal resistant microorganisms do not arise by chance and, that there must be selection factors like environmental contamination by heavy metals (Hideomi et al., 1977). Ecological implications of increased mercury tolerance/resistance observed in this study could mean higher rates of biotransformation of toxic heavy metals; their higher mobilization through marine food web and increased levels of Hg^0 in the atmosphere. As prokaryotic metabolic pathways dealing with elemental mercury (Smit et al., 1998) or its many inorganic salts generally lead to production of more toxic forms (e.g., alkylmercury), consequences of enhanced atmospheric Hg^0 could bring about highly undesirable environmental changes.

The results achieved in the present research show that the waste water effluents of RSP,Rourkela are highly polluted with mercury. Isolated bacteria from these sites showed high levels of resistance to mercury. According to the results of this study it is suggested that mercury resistant bacteria are being isolated with primary enrichment

method in the presence of Hg. Mercury resistant bacteria isolated from contaminated environments have high potential to remove Hg from factory effluents. So it is suggested that mercury elimination ability of these bacteria should be evaluated. Moreover we can genetically engineer these isolates to reach better results in removal of Hg. The metal resistant strains isolated from the waste water sample can be used for bioremediation process by construction of bioreactors where the strains can be immobilized for treating waste water effluents from industrial or domestic sources. Further selection of strains can result in isolation of strains with higher resistance which could serve as an effective means of treating waste water.

Future perspectives

- These isolates can be genetically engineered to reach better results in Hg removal.However before exploiting the strain as an efficient Biotechnological tool which can be used for mercury detoxification, furher investigatory studies needs to be carried in laboratory scale and in situ metal reduction potentiall of genus has to be assayed.

- Enumeration of MRB on a regular basis should be carried out along with microbiological analysis and this can become a regular parameter to realize the health status of environment.In principle,higher their abundance,more likely is the concentration of the heavy metal in the environment.

- Resistance of MRB to other heavy metals to be deciphered and used as a practical means for environmental clean up.

- Presence of non mer mediated Hg volatilization in bacteria may prove pivotal in acquiring more information on Hg resistance.

8. REFERENCES

Amy P.S. and Morita R. Y. (1983). Starvation-survival patterns of sixteen freshly isolated open-ocean bacteria. Appl. Environ. Microbiol. 45:1109- 15.

Baldi F., Filippelli M. and Olson G. J. (1989). Biotransformation of mercury by bacteria isolated from a river collecting cinnabar mine waters. Microb. Ecol. 17: 263-74.

Baldwin D. R. and Marshall W. J. (1999). Heavy metal poisoning and its laboratory investigation. Ann. Clin. Biochem. 36: 267-300.

Barbieri P., Galassi G. and Galli E. (1989). Plasmid-encoded mercury resistance in a *Pseudomonas stutzeri* strain that degrades o-xylene. FEMS Microbiol. Ecol. 62: 375-84.

Barbieri P., Bestetti G., Reniero D. and Galli E. (1996). Mercury resistance in aromatic compound degrading *Pseudomonas* strains. FEMS Microbiol. Ecol. 20: 185-94.

Barkay T. and Olson B. H. (1986). Phenotypic and genotypic adaptation of aerobic heterotrophic sediment bacterial communities to mercury stress. Appl. Environ. Microbiol. 52: 403-06.

Barkay T. (1987). Adaptation of aquatic microbial communities to Hg2+ stress. Appl. Environ. Microbiol. 53: 2725-32.

Barkay T. and Pritchard P. H. (1988). Adaptation of Aquatic Microbial Communities to Pollutant Stress. Microbiol. Sci. 5(6): 165-69.

Barkay T., Liebert C. and Gillman M. (1989). Environmental significance of the potential for mer (Tn21)-mediated reduction of Hg2+ to Hg0 in natural waters. Appl. Environ. Microbiol. 55: 1196-202.

Barkay T., Turner R., Saouter E. and Hron J. (1992). Mercury biotransformation and their potential for bioremediation of mercury contamination. Biodegradation. 3: 147-59.

Barkay T., Gillman M. and Turner R. R. (1997). Effects of dissolved carbon and salinity on bioavailability of mercury. Appl. Environ. Microbiol. 63: 4267-71.

Barkay T. The mercury cycle. In Encyclopedia of microbiology, 2nd ed. Academic press, Inc., San Diego. (2000). Pp. 171-81.

Barkay T., Miller S. M. and Summers A. O. (2003). Bacterial mercury resistance from atoms to ecosystems. FEMS Microbiol. Rev. 27: 355-84.

Bizily S. J., Rugh C. L., Summers A. O. and Meagher R. B. (1999).Phytoremediation of methylmercury pollution: merB expression in *Arabidopsis thaliana* confers resistance to organomercurials. Proc. Nat. Acad. Sci. 96: 6808-6813.

Bizily S. P., Rugh C. L. and Meagher R. B. (2000). Phytodetoxification of hazardous organomercurials by genetically engineered plants. Nature Biotechnol. 18: 213-17.

Blair W. R., Olson G. J., Brinckman F. E. and Iverson W. P. (1982). Accumulation and fate of tri-n-butyltin cation in estuarine bacteria. Microb. Ecol. 8: 241-45.

Bogdanova E. S., Mindlin S. Z., Pakrova E., Kocur M. and Rouch D. A. (1992). Mercuric reductase in environmental Gram-positive bacteria sensitive to mercury. FEMS Microbiol. Lett. 97: 95-100.

Boudou A. and Ribeyre. Mercury in the food web: Accumulation and transfer mechanisms. *In* Sigel A. and Sigel H. (eds), metal ions in biological systems: mercury and its effects on environment and biology. Vol. 34. Marcel Dekker Inc., New York. (1997). Pp. 503-568.

Bradford M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72: 248–54.

Brown N. L., Misra T. K., Winnie J. N., Schmidt A., Seiff M. and Silver S. (1986). The nucleotide of the mercuric resistance operon of plasmid R100 and transposon Tn501: further evidence for *mer* genes, which enhance the activity of the mercuric ion detoxification system. Mol. Gen. Genet. 202: 143-51.

Brown N. L., Camakaris J., Lee B. T., Williams T., Morby A. P., Parkhill J. and Rouch D. A. (1991). Bacterial resistance to mercury and copper. J. Cell. Biochem. 46: 106-14.

Brunke M., Deckwer W. D., Fritschmuth J. M., Horn H., Lunsdorf M., Rhode M., Rohricht M., Timmis K. N. and Weppen P. (1993). Microbial retention of mercury from waste systems in a laboratory column containing merA gene bacteria. FEMS Microbiol. Rev. 11: 45-52.

Canstein V. H., Li Y., Timmis K. N., Deckwer W. D and Wagner-Döbler I. (1999) Removal of mercury from chloralkali electrolysis wastewater by a mercury-resistant *Pseudomonas putida* strain. Appl. Environ. Microbiol. 65: 5279-84.

Canstein H. V. F. (2000). Ph. D thesis "Mercury-reducing biofilms: insights in their activity and composition and application for bioremediation of mercury-polluted wastewaters. Technical University of Braunschweig, Germany.

Canstein V. H., Li Y. and Wagner-Döbler I. (2001). Long-term performance of bioreactors cleaning mercury-contaminated wastewater and their response to temperature and mercury stress and mechanical perturbation. Biotechnol Bioeng. 74: 212-19.

Canstein V. H., Li Y., Leonhäuser J., Haase E., Felske A., Deckwer W. D. and Wagner-Döbler I. (2002). Spatially Oscillating Activity and Microbial Succession of Mercury-Reducing Biofilms in a Technical-Scale Bioremediation System. Appl. Environ. Microbiol. 68: 1938-46.

Colwell R. R., Brayton P. R., Grimes D. J., Roszak D. B., Huq S. A. and Palmer L. M. (1985) Viable, but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. Biotechnology. 3: 817-20.

Condee C. W. and Summers A. O. (1992). A mer-lux transcriptional fusion for real-time examination of in vivo gene expression kinetics and promoter response to altered superhelicity. J. Bacteriol. 174: 8094-101.

Förstner U., Wittmann G. T. W. Metal pollution in the aquatic environment. Springer Verlag Berlin Heidelberg New York. (1979). Pp. 486.

Frischmuth A., Weppen P. and Deckwer W. D. (1991).Quecksilberentfernung aus wässerigen Medien durch aktive mikrobielle Prozesse. Bioengineering. 7: 38.

Fukuda Y., Ushijima K., Sakamoto M. and Futatsuka M. (1999). An analysis of subjective complaints in a population living in a methylmercury-polluted area. Env. Res. 81: 100-07.

Garcia E. M., Orta M. and Suarez P. (1999). Toxicity assays and bioconcentration of mercury in bacteria selected from marine environments. Bull. Environ. Contam. Toxicol. 62: 79-86.

Gerlach A. S. Marine Pollution. Diagnosis and Therapy. Springer-Verlag Berlin Heidelberg New York. (1981). Pp. 218.

Griffin H. G., Foster T. J., Silver S. and Misra T. K. (1987). Cloning and DNA sequence of the mercuric and organomercurial resistance determinates of plasmid pDU1358. Proc. Natl. Acad. Sci. USA. 84: 3112-16.

Hobman J. L. and Brown N. L. Bacterial Mercury-Resistance Genes. In H. Sigel and A. Sigel (eds.). Metal Ions in Biological Systems Vol. 34. Marcel Dekker Inc. New York, USA. (1997). Pp. 527-568.

Hobman J., Kholodii G., Nikiforov V., Ritchie D. A., Strike P. and Yurieva O. (1994). The sequence of the *mer* operon of p327/419, 330 and 05. Gene. 277: 73-78.

Horvat M., Covelli S., Faganeli J., Logar M., Mandiz V., Rajar R., Zirca A. and DuZan . (1999). Mercury in contaminated environments; a case study: the Gulf of Trieste. Sci. Tot. Env. 237/238: 43-56.

Hütter L. A. (1978). Quecksilbergefährdung durch Leuchtstofflampen? Naturwiss Rundsch 3a: 17-19.

Izaki K. (1981). Enzymatic reduction of mercurous and mercuric ions in *Bacillus cereus*. Can. J. Microbiol. 27: 192-97.

Ji G. and Silver S. (1995). Bacterial resistance mechanisms for heavy metals of environmental concern. J. Ind. Microbiol. 14: 61-75.

Kiyono M., Omura T., Inuzuka M., Fujimori P. and Pan-Hou H. (1997). Nucleotide sequence and expression of the organomercurial-resistance determinants from a *Pseudomonas* K-62 plasmid pMR26. Gene. 189: 151-57.

Kiyono M. and Pan-Hou H. (1999). The merG Gene Product is Involved in Phenylmercury Resistance in *Pseudomonas* Strain K-62. J. Bacteriol. 181: 726-30.

Kiyono M., Uno Y., Omura T. and Pan-Hou H. (2000). Involvement of merB in the expression of the pMR26 mer operon induced by organomercurials. J. Hlth. Sci. 46: 142-45.

Kiyono M., Omura H., Omura T., Murata S. and Hou-Pan H. (2003). Removal of inorganic and organic mercurials by immobilized bacteria having mer-ppk fusion plasmids. Appl. Microbiol. Biotechnol. 62: 274-78.

Knight C., Kaiser J., Lalor G. C., Robotham H. and Witter J. V. (1997). Heavy metals in surface water and stream sediments in Jamiaca. Environ. Geochem. Hlth. 19: 63-66.

Kogure K., Simidu U, Taga N. and Colwell R. R. (1987). Correlation of direct viable counts with heterotrophic activity for marine bacteria. Appl. Environ. Microbiol. 53: 2332-37.

Komura I. and Izaki K. (1971). Mechanism of mercuric chloride resistance in microorganisms. I. Vaporization of a mercury compound from mercuric chloride by multiple drug resistance strain of *Escherichia coli*. J. Biochem. 70: 885-93.

Kovalic W. Perspective on risks of soil pollution and experience with innovative remediation technologies. Strategies 2000 Proc. World Congr. Chem. Eng. 4th ed. (1991). Pp. 281-95.

Kusano T., Ji G., Inoue C. and Silver S. (1990). Constitutive synthesis of a transport function encoded by the *Thiobacillus ferrooxidans mer*C gene cloned in *Escherichia coli*. J. Bacteriol. 172: 2688-92.

Lacerda L. D. and Pfeiffer W. C. (1992). Mercury from gold mining in Amazon environment – an overview. Quimica Nova. 55: 238-94.

Langford N. J. and Ferner R. E. (1999). Toxicity of mercury. J. Hum. Hypertension. 13: 651-56.

Liebert C. and Barkay T. (1988). A direct viable counting method for measuring tolerance of aquatic microbial communities to Hg2+. Can. J. Microbiol. 34: 1090-95.

Liebert C. A., Wireman J., Smith T. and Summers A. O. (1997). Phylogeny of mercury resistance (mer) operons from gram-negative bacteria isolated from the fecal flora of primates. Appl. Environ. Micobiol. 63: 1066-76.

Liebert C. A., Hall R. M. and Summers A. O. (1999). Transposon Tn21, flagship of the floating genome. Microbiol. Mol. Biol. Rev. 63: 507-22.

Lund P. A., Ford S. J. and Brown N. L. (1986). Transcriptional regulation of the mercury-resistance genes of transposon Tn501. J. Gen. Microbiol. 132: 465-80.

Lund P. A. and Brown N. L. (1987). Role of the merT and merP gene products of transposon Tn501 in the induction and expression of resistance to mercuric ions, Gene. 52:207-14.

Macdonald R. W., Barrie L. A., Bidleman T. F., Diamond M. L., Gregor D. J. Semkin R. G., Strachan W. M., Li Y. F., Wania F., Alaee M., Alexeva L. B., Backus S. M., Bailey R., Bewers J. M., Gobeil C., Halsall C. J., Harner T., Hoff J. T., Jantunen L. M., Lockhart W. L., Mackay D., Muir D. C., Pudykiewicz J., Reimer K. J., Smit J. N. and Stern G. A. (2000). Contaminants in the Canadian Arctic: 5 years of progress in understanding sources, occurrences and pathways. Sci. Tot. Env. 254: 93-234.

MacFaddin F. J. Biochemical tests for identification of medical bacteria. Williams and Wilkins (eds.). Baltimore, 2nd edition. (1980). Pp. 527.

McArthur J. V. and Tuckfield R. C. (2000). Spatial patterns in among stream bacteria: effects of industrial pollution. Appl. Environ. Microbiol. 66: 3722-26.

McGraph R. (1995). Managing sediment remediation costs. Pollution Engineering. 27, 34-35.

Miller S. M., Ballou D. P., Massey V., Williams C. H. Jr. and Walsh C. T. (1986). Two-electron reduced mercuric reductase binds Hg (II) to the active site of dithiol but does not catalyze Hg (II) reduction. J. Biol. Chem. 261: 8081-84.

Misra T. K., Brown N. L., Fritzinger D. C., Pridmore R. D., Barnes W. M., Haberstroh L. and Silver S. (1984). The mercuric-ion resistance operon of plasmid R100 and transposon Tn501: the beginning of the operon including the regulatory region and the first two structural genes. Proc. Natl. Acad. Sci. USA. 81: 5975-79.

Misra T. K. (1992). Bacterial resistance to inorganic mercury salts and organomercurials. Plasmid. 27: 4-16.

Morby A. P., Hobman J. L. and Brown N. L. (1995). The role of cysteine residues in the transport of mercuric ions by the Tn501 MerT and MerP mercury-resistance proteins. Mol. Microbiol. 17: 25-35.

Moreira J. C. and Piveta F. (1997). Human and environmental contamination by mercury from industrial use in Brazil. Water Air Soil Pollut. 97: 241-46.

Morel F. M. M. and Hering J. G. Principles and Applications of Aquatic Chemistry. Wiley-Interscience, New York. (1993). Pp. 588.

Morel F. M. M, Kraepiel A. M. L. and Amyot M. (1998). The chemical cycle and bioaccumulation of mercury. Ann. Rev. Ecol. Syst. 29: 543-66.

Müller A. K., Rasmussen L. D. and Sorensen S. J. (2001a). Adaptation of the bacterial community to mercury contamination. FEMS Microbiol. Lett. 16: 49-53.

Müller K. A., Westergaard K. and Christensen S. (2001b). The effect of long-term mercury pollution on the soil microbial community. FEMS Microbiol. Ecol. 36: 11-19.

Nakamura K., Fujisaki T. and Tamashiro H. (1986). Characteristics of Hgresistant bacteria isolated from Mina Mata Bay sediment. Environ. Res. 40: 58-67.

Nakamura K. and Nakahara H. (1988a). Simplified x-ray film method for detection of bacterial volatilization of mercury chloride by *Escherichia coli*. Appl. Env. Microbiol. 54: 2871-73.

Nakamura K., Fujisaki T. and Shibata Y. (1988b). Mercury-resistant bacteria in the sediment of Minamata Bay. Nippon Suisan Gakkaishi. 54: 1359-63.

Nakamura K., Mineshi S., Uchiyama H. and Yagi O. (1990). Organomercurial volatilizing bacteria in the mercury polluted sediment of Minamata Bay, Japan. Appl. Environ. Microbiol. 56: 304-05.

Nascimento A. M. A. and Chartone-Souza E. (2003). Operon mer: bacterial resistance to mercury and potential for bioremediation of contaminated environments. Genet. Mol. Res. 2: 92-101.

Nriagu J. O. (1990). Global metal pollution. Poisoning the biosphere? Environment. 32: 7-33.

Nucifora G., Chu L., Silver S. and Misra T. K. (1989). Mercury operon regulation by the merR gene of the organomercurial resistance system of plasmid pDU1358. J. Bacteriol. 171: 4241-47.

Osborn A. M., Bruce K. D., Strike P. and Ritchie D. A. (1993). Polymerase chain reaction-restriction fragment length polymorphism analysis shows divergence among mer determinants from gram-negative soil bacteria indistinguishable by DNA-DNA hybridization. Appl. Environ. Microbiol. 59: 4024-30.

Osborn A. M., Bruce K. D., Strike P. and Ritchie D. A. (1997). Distribution, diversity and evolution of the bacterial mercury resistance (mer) operon. FEMS Microbiol. Rev. 19: 239-62.

Pahan et al (1990). "Ecological and Biochemical studies on Mercury resistant bacteria". Ind. J. Env. Hlth. 32: 250-61.

Radstrom P., Skold G., Swedberg G., Flensburg J., Roy P. H. and Sundstrom L. (1994). Transposon Tn5090 of the plasmid R751, which carries an integron, is related to Tn7, Mu, and the retroelements. J. Bacteriol. 176: 3257-68.

Rai L. C., Malick N., Singh J. .B. and Kumar H. D. (1991) Physiological and biochemical characterstics of a copper tolerant and a wild type strain in *Anabaenaa doliolum* under copper stress. J. Plant Physiol. 38: 68-74.

Ramaiah N. Ph.D thesis "Studies on Marine Luminous Bacteria". November (1989). Pp. 230.

Ramaiah N. and Chandramohan D (1993). Ecological and laboratory studies on the role of luminous bacteria and their luminescence in coastal pollution surveillance. Mar. Poll. Bull. 26: 190-201.

Ramaiah N., Kenkre V. D. and Verlekar X. N. (2002a) Marine environmental pollution stress detection through direct viable counts of bacteria. Wat. Res. 36: 2383-93.

Ramaiah N., Ravel J., Straube W. L. and Colwell R. R. (2002b). Entry of *Vibrio harveyi* and *Vibrio fischeri* into the viable but nonculturable state. J. Appl. Microbiol. 93: 108-16.

Ramaiah N. and De J. (2003). Unusual rise in mercury-resistant bacteria in coastal environs. Microb. Ecol. 45: 444-54.

Rasmussen L. D. and Sørensen S. J. (1998). The effect of longterm exposure of mercury in the bacterial community in marine sediment. Curr. Microbiol. 36: 291-97.

Rasmussen L. D. and Sorensen S. J. (2001). Effects of mercury contamination on the culturable heterotrophic, functional and genetic diversity of the bacterial community in soil. FEMS Microbiol. Ecol. 36: 1-9.

Reyes N. S., Frischer M. E. and Sobecky P. A. (1999). Characterization of mercury resistance mechanisms in marine sediment microbial communities. FEMS Microbiol. Ecol. 30: 273-84.

Robinson J. B. and Tuovinen O. H. (1984). Mechanisms of microbial resistance and detoxification of mercury and organomercury compounds: physiological, biochemical and genetic analysis. Microbiol. Rev. 48: 95-124.

Rugh C. L., Senecoff J. F., Meagher R. B. and Merkle S. A. (1998). Development of transgenic yellow poplar for mercury phytoremediation. Nature Biotechnol. 16: 925-28.

Sant'ana Y. X., Chartone-Souza E. and Ferreira M. D. (1989). Drug resistance and colicinogeny of *Salmonella typhimurium* strains isolated from sewage-contaminated surface water and humans in Belo Horizonte, Brazil. Rev. Microbiol. 20 : 41-49.

Schwarzenbach R. P., Gschwend D. M. and Imboden D. M. Environmental Organic Chemistry. New York, John Wiley & Sons, Inc. (1993). Pp. 681.

Selifonova O. V. and Barkay T. (1994). Role of Na+ in transport of Hg2+ and induction of the Tn21 mer operon. Appl. Environ. Microbiol. 60: 3502-07.

Sharma P. K., Blackwill D. L., Frenkel A. and Vairavamurthy M. A. (2000). A new *Klebsiella planticola* strain (Cd-1) grows anaerobically at high cadmium concentrations and precipitates cadmium sulfide. Appl. Environ. Microbiol. 66:3083-87.

Silver S. (1996). Bacterial resistances to toxic metals- a review. Gene. 179: 9-19.

Silver S. and Misra T. K. (1988). Plasmid-mediated heavy metal resistance. Ann. Rev. Microbiol. 42: 717-43.

Silver S., Endo G. and Nakamura K. (1994). Mercury in the environment and laboratory. J. Japan. Soc. Water Envrion. 17: 235-44.

Silver S. and Phung Le T. (1996). Bacterial heavy metal resistance: New Surprise. Annu. Rev. Microbiol. 50: 753-89.

Slemr F., Brunke E. G., Ebinghaus R., Temme C., Munthe J., Wängberg I., Schroeder W. and Steffen A. (2003). Worldwide trend of atmospheric mercury since 1977. Geophys. Res. Lett. 30: 1516-18.

Soldano B. A., Bien P. and Kwan P. (1975). Air-borne organomercury and elemental mercury emissions with emphasis on central sewage facilities. Atmos. Environ. 9: 941-44.

Strasdeit H. (1998). Methylquecksilber: eine Warnung. Nachrichten aus Chemie, Technik und Laboratorium. 46(9): 846-48.

Summers A. O. and Silver S. (1972). Mercury resistance in a plasmid-bearing strain of *Escherichia coli*. J.Bacteriol. 112: 1228-36.

Summers A. O. and Lewis E. (1973). Volatilization of mercuric chloride by mercury-resistant plasmid-bearing strains of *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. J. Bacteriol. 113: 1070-72.

Summers A. O. and Silver S. (1978). Microbiological transformation of metals. Ann. Rev. Microbiol. 32: 637-72.

Summers A. O. (1986). Organization, expression and evolution of genes for mercury resistance. Ann. Rev. Microbiol. 40: 607-34.

Summers A. O. (1992). The hard stuff: metals in bioremediation. Curr. Opin. Biotechnol. 3: 271-76.

Wagner-Döbler I., Lünsdorf H., Lübbehüsen T., Von Canstein H. F., and Li Y. (2000). Structure and Species Composition of Mercury-Reducing Biofilms. Appl. Environ. Microbiol. 66: 4559-63.Industrial wastewater. Appl. Microbiol. Biotechnol. 62: 124-33.

Wang C. L., Michels P. C., Dawson S. C., Kitisakkul S., Bross J. A., Keasling J. D. and Clark D. S. (1997). Cadmium removal by a new strain of *Pseudomonas aeruginosa* in aerobic culture. Appl. Environ. Microbiol. 63: 4075-78.

Wang Y., Mahler I., Levinson H. S., Silver S. and Halvorson H. O. (1987). Cloning and expression in *Escherichia coli* of chromosomal mercury resistance genes from a *Bacillus* sp. J. Bacteriol. 169: 4848-51.

Wang Y., Moore M. J., Levinson H. S., Silver S. and Walsh C. T. (1989). Nucleotide sequence of a chromosomal mercury resistance determinant from a *Bacillus Strain.*

Xiao Z., Sommar J. and Lindquist O. (1998). Atmospheric mercury deposition on Fangjing mountain nature secvice, Guizhou, China. Chemosphere. 36: 2191-200.